

The Chinese dimension

A MOST remarkable piece of Chinese science is reported on page 286. It seems that as a result of a major redirection in scientific goals during the Cultural Revolution, the Chinese have been able not only to start issuing credible predictions of earthquakes, but also to save lives by firm action. Such success is bound to raise questions about what lessons we could learn from the Chinese, and whether their very distinctive brand of 'science for the people' could with profit be adopted in other countries.

In many ways the Chinese earthquake programme is a unique one. It has been possible to involve amateurs in the reporting process, even to the extent of asking peasants to pass on news of unusual animal behaviour. It has also been possible to rely on an orderly public response to a warning—something very difficult to envisage in southern California. There is something akin to the feeling of a popular crusade in the whole operation; this is hardly a surprise since in the past hundred years at least 200,000 Chinese have been killed in earthquakes (a hundred times the number of American deaths from that cause). But many of those who return from China express considerable concern that the short term advantages of people-involvement will be rapidly squandered if there is no long term policy of upgrading the role of the scholar and scholarship.

Some scholarly facilities are highly praised—particularly libraries—but there are considerable doubts about the adequacy of postgraduate training; indeed in many cases it is almost non-existent. After a three-and-a-half year undergraduate career, the best that the intelligent student can hope for is to be seconded to some field unit where the problems are intellectually demanding and where, at the least, he may pick up some form of scholarship from an older professor with experience of the former style of graduate education. As a means of mobilising a large technical army and convincing the general public that science is working for them, the present method cannot be faulted. It does, however, pose serious problems when it comes to capitalising on genuinely outstanding talents, and there is some doubt that, amidst the welter of activity on every conceivable front, enough criticism goes into the planning of future work.

The American committee, whose report is described on page 286, writes "the local scientific workers described the experiments in which they are engaged. Discussing their work in this manner is apparently an unusual experience for many of these young scientists, and when we questioned or disagreed with them they seemed unaccustomed to the give-and-take of scientific criticism which is so characteristic of the American scientific establishment . . ." As a result, it seems that the Chinese are ever-enthusiastic to measure anything and to try any longshot, regardless of whether or not there is any sound scientific justification for it. In some cases this could pay off handsomely (it is possible that in monitoring animal behaviour the Chinese could be on to something big), but the pay-off is more likely to be in the West, which is better intellectually and organisationally equipped to take anomalous observations, build, refine and use models. It is to be hoped that delegations travelling out of China have picked up some ideas, too, on the running of science.

One of the intriguing questions that arises from the several tens of exchanges of scholarly delegations in the past two years is whether the obvious interest in China is going to be at the expense of exchanges with the Soviet Union. This is partly a political question, of course, and depends on the future of the triangular relationship between Peking, Moscow and Washington. There is, however, little doubt that if the decision were left to the scientists, many would like to cultivate their Chinese contacts, even though the many close friendships that westerners have with individual Soviet scientists would probably not be paralleled in the more anonymous Chinese situation.

There are perhaps two non-scientific reasons why China is appealing—it has an extraordinarily rich cultural scene, and there is an apparent lack of the endless feeling of being watched which has made so many westerners almost relieved to get out of the Soviet Union. But what excites visitors scientifically is the feeling that Chinese science is involved in a great experiment in mutual help; those who do not have their minds ideologically closed want, in some fashion, to see the outcome of the experiment and even to help participate in it. □



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bred crops, however, is restricted by comparison with that represented by the older varieties. The widespread introduction of new strains into hitherto undisturbed areas of genetic diversity, such as the massive introductions of the Mexican-bred semi-dwarf wheats into India and the Near East, actually poses a grave threat to the continued existence of many primitive strains, which are now found only sporadically and in remoter regions.

Where the introduced crops mean higher yields and a better standard of life for the farmer, the disappearance of the older varieties from the fields is inevitable. But the potentially valuable genes which they contain must be preserved, for they will certainly be needed in future crop breeding programmes. Nobody yet knows the full extent of variation available in traditional crops, and many qualities which could be overlooked now might be the very ones desperately needed in the future.

Although the disappearance of traditional varieties is unlikely to slow down, for many crops a reasonably representative sample of the variation in any population can be conserved easily and economically as seed. Seeds of many species remain viable for years if they are kept dry and cold. The optimum conditions for storing many types of seed are now well-known, and gene banks for most important crops have been established in centres all over the world. The old varieties and their wild relatives have been a fruitful source of genes resistance for pests and disease; moreover, they will always have an important part to play in the never-ending race to breed new resistant varieties before the pests themselves progress.

Unusual cultural characteristics sometimes found in primitive varieties have had a revolutionary effect on plant breeding. The semi-dwarf habit in rice, which formed one of the basic traits of the rices of the Green Revolution, was introduced from a primitive variety found in Taiwan. Scientists at the International Rice Research Institute in the Philippines, where the first dwarf rices were bred, are now attempting to combine high yield with the 'floating' characteristic of rices grown for generations in the deep water rice areas around the great rivers of India and South-east Asia.

International efforts to conserve the rapidly vanishing reservoir of genetic diversity began some 15 years ago when the Food and Agriculture Organisation of the United Nations (FAO) convened a technical meeting on plant introduction and exploration. In 1963 an FAO panel of experts on plant exploration and introduction was established, and in 1968 the Crop

Future in the past

Modern agriculture threatens to destroy the very means by which it developed. The recent establishment of the International Board for Plant Genetic Resources will help to coordinate international efforts to conserve the rapidly diminishing genetic resource represented by traditional varieties of crops grown for generations. Eleanor Lawrence looks at some of the projects now under way.

MOST of the staple crops grown today were first brought under cultivation thousands of years ago. Cultivated plants have spread out from their original centres of cultivation until they can be found today growing in conditions very different from those in which they originated. In the process they have diversified into innumerable genetically different local varieties, each showing some adaptation to the climate and general environment in which they now flourish.

This reservoir of genetic raw material has in the past been of inestimable value to the plant breeder. Over thousands of years of evolution and selection, genes for resistance to many threatening and economically damaging pests and diseases have arisen. Useful qualities such as cold and drought tolerance have developed in varieties growing at the limits of their range. Plant breeders have constantly returned to the older strains for useful genes which can often be found only in a very few varieties or in their wild relatives. Now, however, this invaluable genetic resource is fast disappearing as a result of replacement of traditional crops by the generally more productive newer varieties.

The small fields of primitive agri-

culture bear little resemblance to the monocultures of industrialised developed countries. Several varieties of the same crop are often grown together, and the individual plants differ greatly from each other. The wild relatives of the cultivated plant may well be present either as weeds in the field or growing along pathways and field margins.

This variability sometimes results in relatively low yields. Indeed, peasant farmers have often selected for reliability rather than high performance, on the basis that in a bad year a small but dependable yield is better than no yield at all. But it is the genetic mixture itself which makes possible the emergence of desirable new forms by fortuitous crossbreeding. These forms may then be selected simply by their superior ability to cope with local conditions, or directly by the farmer.

Agriculture in the developed countries, where intensive plant breeding has been practised, now depends on a comparatively few varieties of the major crops. When grown with modern aids, such as fertilisers, pesticides and herbicides, these produce yields far greater than those possible with the older highly diverse 'landraces'. The gene pool represented by these highly

Primitive wheat in Afghanistan, showing variability (FAO photo by E. Bennett)

Ecology and Genetic Resources Unit, a special section within the FAO, was set up to act as a clearing house for information on genetic resources and to administer the FAO's programme.

The interest this aroused confirmed that the problem was as grave as had been feared. Moreover, the efforts already underway by individual institutes and the FAO itself were only able to tackle the most urgent problems. A relatively large amount of material has now been collected from the more accessible areas, but remoter regions of the world, which contain much of the remaining genetic diversity in many major crops, remain largely unexplored.

A plan for a worldwide network of genetic resource centres incorporating existing institutes and projects within an international collaborative programme was therefore drawn up at a meeting in 1972 sponsored by three bodies: the FAO, the Consultative Group on International Agricultural Research (CGIAR) and the Technical Advisory Committee to the CGIAR.

The programme is coordinated by a committee created 18 months ago, the International Board for Plant Genetic Resources (IBPGR). This falls under the aegis of CGIAR and has a secretariat in the Crop Ecology and Genetic Resources Unit of the FAO. Its budget in 1975 was some \$730,000, and a budget of \$1.3 million has been proposed for 1976. A year of fact-finding has resulted in several projects now being undertaken with IBPGR funds.

One of the most important projects from the point of view of coordination and information exchange is the genetic resources communication, information and documentation system (CIDS) project based at the University of Colorado at Boulder. This project is intended to realise the full potential of the collections made, which means they must not only be described botanically, but in such a way that the plant breeder can easily identify material which might be useful in a breeding programme. Botanists and plant breeders who wish to use the materials must know details of the original habitat, morphology and local conditions, which can only be recorded at the time of collection. Information on disease resistance and physiological properties can be added later after proper evaluation.

The CIDS project was originally set up, and is still partly supported, by the Crop Ecology and Genetic Resources Unit of the FAO, with the aim of devising a computer system suitable for recording and retrieving information on crop plant genetic resources. The IBPGR has given its support, allocating \$237,000 to the project in 1975. It hopes to provide another \$360,000 in 1976.

Through visits to existing collections and intensive collaboration with both collectors and plant breeders, the CIDS team has initiated a transfer of genetic resources records into a storable form through a system (TAXIR/EXIR) which is adaptable to any of the principal types of computer with an adequate memory bank. It is hoped that eventually the inventories of all major collections will be filed on this or some other compatible system, which will allow the user to retrieve the collections from the area or with the particular characteristics he wants.

As for the future, some of the field projects which the IBPGR is to finance out of its own fund for 1976 include the Near-East Regional Programme, a programme for the Mediterranean area centred on the Germplasm Laboratory at Bari in Italy, and the development of a regional programme for south and south-east Asia. The Board is also financing collections of rice by the International Rice Research Institute (IRRI) in Bangladesh, Indonesia and other south-east Asian countries.

Many important tropical forage legumes originate in Latin America—the pastures which support the Australian livestock industry are made up almost entirely of introduced species, many from Latin America—and the Board has also provided funds to begin a three-year programme of collection, conservation and evaluation of tropical forage legumes and grasses there. This work is to be carried out by the Centro Internacional de Agricultura Tropical (CIAT) in Colombia.

Attempts to improve the natural pastures in Latin America have used native legumes such as *Stylosanthes*, *Centrosema*, *Desmodium* and *Macropitium* which make important contributions to the nitrogen fixing ability of the pasture and its nutritional value. But the improvement has been at best sporadic, and the CIAT programme

aims to make a systematic and representative collection of these tropical forage legumes and their accompanying symbiotic bacteria, the *Rhizobia*, as well as some tropical grasses.

Ethiopia is an immensely important region of genetic diversity under stress and is an especially important area of variation in wheat and barley. Although several collecting expeditions have been mounted over the years, by the Germplasm Laboratory at Bari for instance, the region warrants its own regional genetic resources centre, like that serving the Near-East at Izmir in Turkey. The Federal Republic of Germany offered to finance such a centre several years ago, and the first funds are to be spent in 1976.

Natural disasters as well as human activity can threaten genetic resources. An expedition organised by the French Organisation for Scientific Research Overseas (ORSTROM), financed by the United Nations Environment Programme, is now in the Sahel collecting as much as possible of the remaining native varieties of sorghum and millet. The indigenous varieties are particularly resistant to drought, a characteristic not found elsewhere. After a disaster, restocking tends to be carried out with easily available varieties brought in from outside which lack the drought resistant characteristics peculiar to the indigenous varieties. If the native genetic material can be collected and stored, however, it can be used in future breeding programmes and even be reintroduced if such a disaster strikes again.

With so much to be done, the limited funds of the IBPGR are to be used mainly as 'seed' money to start off some urgently needed programmes. Its other task is to identify neglected areas and encourage potential donors to put their money into the most worthwhile projects—a task which will ultimately prove to be equally important.

Information retrieved from genetic resources data bank

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ONCE upon a mountain there was a man named John Muir. He lived on breadcrusts and tea, and he slept in a blanket beneath the shelter of stunted trees (*Pinus albicaulis*) at timberline. He became famous because he awakened the minds of many people to the need for preserving and protecting the wild meadows, flowers, forests, streams and lakes of the Sierra Nevada and other wildernesses.

But, incredibly, Muir's crusade to save the wilderness gave birth to an organization that became a replica of Big Business. Other groups followed suit. The metamorphosis was an evolutionary process; success attracts money, money employs people, and then the payroll must be met, and, above all, the organization must grow. With fascination, I have watched the change in John Muir's legacy—the Sierra Club. He used to hike with the Club on their annual summer outing to the mountains that started in 1901 and has continued through the years.

For the first fifty years, the outings were on a modest scale. In 1957, the cost of the 28 scheduled trips totalled \$1,571, an average of \$4 per person per day; and obviously cheaper than staying at home. Then came the boom. In 1972, there were 208 trips, and some cost as much as \$1,725 each for five weeks, not including jet plane fares. Apparently the Sierra Club has not reached its Limits to Growth.

With growth comes financial responsibility. Those who are engaged in saving the world need money, but they are too busy with important matters to concern themselves with investment.

Professional counsellors handle such nagging details. Fortunately, there is a Freedom of Information act that allows such matters to be uncovered. The National Audubon Society, once a birdwatchers' club, has a handsome and diverse portfolio of securities including

Eco-business



THOMAS H. JUKES

Strathmore Paper Co., Inland Steel, General Electric, Atlantic Richfield Oil, Walt Disney Productions, Texaco, and Columbia Gas. Moreover, Audubon, the rescuer of birds from slicks and spillage made by the polluting oil companies, has a nice gas and oil business in its Rainey Wildlife Sanctuary in Louisiana which brings in \$300,000 annually.

Great huffing and puffing followed these disclosures. The President of Audubon, who used to be Secretary of the Army, said that the Society needed experience in actually producing gas and oil. A police court magistrate, I suppose, should commit a few burglaries to get the feel of the thing. The Sierra Club Foundation is long on Exxon, General Motors, Tenneco, Public Service of Colorado (nuclear reactors), strip-mining firms and pulp-mill companies. The Environmental Defence Fund defended its fiscal environment in 1973 by cashing dividend cheques from Mallinckrodt Chemical, Avon Products, Exxon, and Philip Morris.

Some security analysts say that the relationship of conservationists to petroleum companies is symbiotic, citing the case of the Alaskan Pipeline. The construction of this was delayed because of a campaign, spearheaded by the Sierra Club, after a large stock of steel pipe had been purchased in Japan and delivered to the frozen tundra before the current inflation. The price of crude oil zoomed up during the delay, and now the oil companies are using the low-cost pipe, which will transport oil whose net worth multiplied *in situ* while conservationists fought valiantly on behalf of the caribou . . .

I am told that caribous have developed a habit of sleeping on the wooden drilling platforms in Alaska, apparently to ward off the chill of the permafrost. Oh, well!—John Muir rolled himself in a blanket. May as well keep warm! □

international news

To help avert "mass hunger for future decades", there should be an immediate, massive infusion of funds into research on food production and a major overhaul of the agricultural research system in the United States, according to two reports submitted last week to President Ford by the National Academy of Sciences. The reports outline preliminary findings of a two-year study, due to be completed in June 1977, of how research in the United States can help combat the grim prospect of increasing hunger and starvation throughout the world.

Specifically requested by President Ford in response to the World Food Conference, which was convened in Rome a year ago, the reports are surprisingly critical of agricultural re-

A thought for food

by Colin Norman, Washington

search policies in the United States. Although there have been spectacular increases in domestic agricultural production during the past three or four decades—to the point, in fact, where North America is now virtually the world's breadbasket—the reports suggest that research efforts lack central coordination, receive insufficient funds, and pay too little attention to basic studies. The preliminary reports, which are designed to influence the Ford Administration's budget for next year

(which is now being prepared), thus offer a prescription for improving the research system, and suggest a number of specific projects requiring an immediate injection of funds.

One of the reports, prepared by a committee headed by Dr Harrison Brown of California Institute of Technology (*World Food and Nutrition Study: interim report*) presents an overall view of how agricultural research in the United States can assist in coping with the world food problem. It draws heavily on the findings and recommendations of the second report, concerned mostly with domestic agricultural research policy (*World Food and Nutrition Study: Enhancement of Food Production in the United States*), which was prepared by a committee

headed by Dr Sylvan Wittwer of Michigan State University.

Although it is tempting to suggest that the world food problem could virtually be solved by better distribution of food supplies, the Brown committee points out that, with world population increasing by about 2.5% a year, better food distribution will offer at best a stop-gap solution. A few summary facts speak for themselves.

Developing countries increased their food production by about 2.8% a year during the 1950s and 1960s, an impressive achievement which reflects both increasing yield from individual farms and increasing acreage under cultivation. But their effective demand for food in the 1960s increased even more sharply, by about 3.5% a year, and it will continue to do so for several decades. The result has been a rapid escalation in food imports by the developing countries—a trend which, according to estimates of the Food and Agriculture Organisation, will result in total cereal imports of 100 million tons by 1985.

The Brown committee therefore argues that “the key to adequate feeding of peoples in the developing countries over the coming decades is a sustained trend of very substantial increases in the yield of their farms”. The increases, in fact, will have to be even more spectacular than those brought about by the recent introduction of high-yielding varieties of wheat and rice—the so called Green Revolution—and the committee also notes that “there is a parallel requirement for continuing rapid expansion of US yields on our principal export crops”.

Such achievements will require “a greatly strengthened research base”, the committee argues, and its recommendations essentially boil down to three requirements: increased participation by research institutions and scientists from the United States in international research and development activities; increased attention to several lines of research in the United States which have worldwide implications; and some overdue reforms in the planning and execution of domestic agricultural policy.

● Proceeding from the observation that “it is not feasible in the next decade or two to meet the developing world’s massive requirements for research capabilities and research results one country at a time”, the committee urges “redoubled US efforts” to help build collaborative international research networks—such as CIMMYT, the International Centre for the Improvement of Maize and Wheat, where Norman Borlaug developed his high-yield wheat varieties. In addition to

increased funding for such networks, the committee also urges greater participation by universities and other institutions within the United States in the work of the international research centres.

● As for research projects which merit increased attention, the Brown committee notes that the “prevailing bias” among politicians and research administrators is toward applied research likely to yield quick results. The Wittwer committee is less charitable: “Fundamental research undergirding food production technology has languished for two decades”, it asserts. “Because of crop surpluses, political pressures from commodity groups, budgetary reductions, and emphasis on immediately applicable results, a formerly substantial basic research effort . . . virtually disappeared”.

The Wittwer committee has consequently drawn up a list of research efforts which have been relatively neglected in the rush for quick results. An expanded research programme on photosynthesis is “urgently needed”, the committee suggests. It recommends that federal support of such activities be doubled, from \$10 to \$20 million a year, and that two or three research institutes be established to spearhead the effort. Similarly, research on nitrogen fixation should receive a large injection of federal funds. “The current funding level of less than \$5 million from all sources in this nation for research on biological nitrogen fixation is grossly inadequate”, the committee states, and suggests that an immediate escalation to \$25 million is needed, followed by a 25% annual increase for the next five years.

Basic genetic research to improve plant yields—including DNA recombination techniques—should also receive a five-fold increase in the present funding level of about \$500,000 a year, the committee urges. Other areas singled out for increased attention are the adaptation of chemical fertilizers for use in tropical regions, improved methods of pest control, ways to combat livestock diseases, and a better assessment of land and water resources in the United States.

The Brown committee accepts the need for increased efforts on those projects, notes that “there is little to be gained from identifying research work that needs acceleration unless US Government food research budgets are increased sharply”, and recommends that expenditures on agricultural research should be increased by at least 50% over the next two or three years.

● Agricultural research in the United States is funded chiefly by block grants to a conglomeration of many federal, state and private research institutions,

and both the Brown and Wittwer committees suggest that it lacks central coordination and direction. It is not the first time that committees of the National Academy of Sciences have criticized the system. Three years ago, in one of the most candid assessments of a federal research effort ever to emerge from the academy, a committee under the chairmanship of Dr Glenn Pound of the University of Wisconsin argued that agricultural research supported by the Department of Agriculture is often pedestrian, badly managed and inefficient.

The Wittwer committee seems to have accepted the thrust of that assessment. It recommends that a top-level National Agricultural Research policy Committee should be established to develop a national research policy. Moreover, the committee states that “many agricultural research administrators are of the opinion that (the Department of Agriculture) has not been an effective proponent of agricultural research, that it does not now provide for adequate consideration of the problems and needs of research in its top-level deliberations, and that it lacks administrative and budgeting arrangements that can effectively guide research in response to national and regional needs”. It therefore suggests a number of administrative changes within the Department of Agriculture designed to coordinate the activities of its fifteen research divisions, and to enhance the status of agricultural research.

As for funding mechanisms, the committee urges that diverse sources of support for agricultural research be brought together. It also suggests that more funds should be available for support of individual research projects, assessed by peer-review panels and supported in the same manner that biomedical research is supported by the National Institutes of Health.

Although some of those suggestions were incorporated into the report of the Brown committee, it promised to give more thought in its final report to the manner in which overall food and nutrition policy should be developed.

Finally, it should be noted that the Brown committee clearly acknowledges that, in dealing only with the limited topic of agricultural research, it necessarily neglects some of the prime considerations in the world food problem, such as population, education and economic planning. Moreover, the committee also states that it does not “assume superior US wisdom on how to achieve development purposes, but only a capability and willingness to handle research problems and to collaborate with others in problem solving where our participation is welcomed”.

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Pesticide perspective

ANOTHER shot was fired last week in the long-raging battle between two of the main schools of thought on the balance to be struck between solutions to the world food problem and their environmental consequences.

Dr Cleve Goring, the director of research and development in plant science for the American-based conglomerate Dow Chemical, nailed his colours firmly to the mast when he spoke at the British Insecticide and Fungicide Conference, held in Brighton.

The three-fold increase in world food production needed by the end of the century to feed the world, he said, would probably require a five-fold increase in the use of pesticides. But he warned that pesticides would, along with other agricultural aids, only help to buy time, rather than solve the long-term food problem.

Dr Goring further warned that "extremists" in the "environmental movement and medical research establishment", especially in the United States, would cause the "unjustifiable elimination" of many products now in use from which the future demand for pesticides would primarily be met. They had to "develop some perspective on the largely imaginary horror of a 'Silent Spring' and the minute amounts of pesticide chemicals that occur in our food".

In speaking out so strongly, Dr Goring drew attention to the apparently increasing plight of even the largest chemical companies engaged in pesticide production. There is evidence that work on new pesticides is being cut not simply as a result of the effects of the recession on research and development but also because the returns are too low for other reasons.

It is accepted even by some environmentalists that the balance is wrong between the time it now takes to develop a new product and the period that the patent laws last. Moreover, it has now become apparent that the new products least likely to yield an economic return are actually the safest in environmental terms because they are also the most selective in their effects.

Dr Goring referred to these matters when he outlined four problems of special concern in the development and use of pesticides:

- The excessive time required in some countries to bring a product from the discovery stage to the market place.
- The need for a "benchmark system" to evaluate the environmental acceptability of new pesticides.
- The lack of agreement between countries on uniform test procedures and tolerances.
- The lack of a workable policy for products reputed to be carcinogenic.

On the increasingly difficult problem of carcinogenicity, Dr Goring had a sharp word for the US Environmental Protection Agency (EPA). He described its recent list of cancer principles as "perhaps the most serious problem", and pointed out that a published list of suspected carcinogens included "many of the natural materials essential to living systems".

But given Dow Chemical's place in the market—sales of pesticides and consumer products last year accounted for more than 10% of its total sales of almost \$5,000 million, making it the eighth largest chemical company in the world—Dr Goring's concern was naturally with what he called "over-regulation" of pesticides, which had advanced "beyond all reason".

He was reflecting the view of those who believe that the chances of further chemical breakthroughs were now being reduced. But this view seems to be about as widespread as the environmentalists' belief that there are enough pesticides in existence already. Between the two, however, is an even more widely argued contention that it is not so much the pesticides themselves which are dangerous as the manner in which they are used. As one expert put it last week, "people need not be against their use, only against dangerous substances carelessly used".

Nuclear deals proliferate

THE path towards nuclear non-proliferation is tortuous and often contradictory. This was revealed again recently when more nuclear deals involving less developed countries were announced at about the same time as further meetings of the Nuclear Suppliers Group took place in London.

The group, whose members include most of the world's nuclear countries—the USA, the USSR, the UK, France, West Germany, Canada and Japan—wishes to prevent the use of fissile waste products for the manufacture of nuclear weapons. But some of its members are also solidly encouraging the use of nuclear power outside their own privileged circle.

In recent months deals have been reported between France and South Korea for a plutonium separation plant, and between West Germany and Brazil for complete nuclear systems including fuel reprocessing. Secret dealings between West Germany and South Africa have also been brought to light.

Now the UK has joined at least six other countries, including Australia and South Africa, which are interested in Iran's ambitious nuclear programme,

and has announced a dual agreement that offers both training and technical assistance, but no reactors.

And the United States apparently promised Egypt two nuclear power reactors, for use in desert desalination plants, during President Sadat's visit to Washington earlier this month. At the same time, the influential Cairo newspaper *Al Ahram* reported that it was Egypt's intention to build 10 nuclear power stations during the next 20 years.

Large as it is, the Egyptian programme barely holds a proverbial candle to that planned in Iran, where the general aim is to make nuclear power the prime source of energy in the country by the 1990s. This will involve some 20 reactors with a combined capacity of around 23,000 MW—more than double Egypt's proposed 10,000 MW, which is its target for the year 2000.

Fears that Iran may be tempted to establish itself as a full nuclear power have not yet manifested themselves as they have over Egypt's plans, in spite of the stimulus that India's nuclear explosion obviously gave to the Shah. The Middle East situation, where Israel's nuclear potential has increased, looms larger in the diplomatic mind, as Dr Kissinger implied when he quickly confirmed that an acceptable arrangement to safeguard the security of the by-products had been worked out with Egypt.

The issue is clearly important to the Nuclear Suppliers Group. The British Foreign Secretary, Mr James Callaghan, emphasised the matter at the UN in September, pronouncing that "the statesmen of the world have a moral duty to act before it is too late to work out the means of controlling the nuclear threat". The talks in London, in fact, were apparently convened in order to discuss the proposals he had made in the General Assembly.

In the meantime, however, the nuclear countries continue to deal among themselves. The British Nuclear Power Company has signed an "enabling agreement" with the Soviet Union on the exchange of experience, techniques and possibly materials connected with the so-called 'steamer' reactors due to come into operation in the UK in the 1980s but already working there. And the efforts to bring Canada's successful technology to the UK continue.

There is evidence of increasing resistance within the nuclear countries to the elaborate nuclear power programmes at home. But so far nothing similar has revealed itself in countries wanting such programmes. Only the Nuclear Suppliers Group is showing signs of concern. But it is only the Nuclear Suppliers Group that is acting to satisfy those wants. □

TEN countries agreed last week on a multi-million pound international programme of five research projects, all based in the UK, designed to develop new technology in the use of coal and so help to solve the energy problem.

The biggest project, costing £10 million, involves the design, construction and operation of an advanced experimental plant near Barnsley which will apply a new technique to coal. It will be financed by the UK, the USA and West Germany.

The technique, known as fluidised combustion because powerful air jets turn burning coal into a bed of red-hot ash that looks and acts like boiling liquid, is said to offer important advantages over conventional steam-raising plant.

Its compactness means a lower capital cost—a big advantage in an era of economic stringency. In addition, it has a high thermal efficiency even with low quality coal, and it cuts pollution by retaining sulphur in the ash bed. It is these advantages which the project is designed to assess.

The other four projects, which cover research services, will together cost about £1 million a year, to be financed by the 10 countries—Austria, Belgium, West Germany, Italy, the Netherlands, Spain, Sweden, Turkey, the UK and the USA.

These aim to estimate the reserves of economically recoverable coal, provide information on coal as an energy resource and assess the costs of new techniques for mining and using coal. A mining technology service will also be established.

The programme is the result of the efforts of the coal technology working group, operating under the aegis of the International Energy Agency. The group is headed by Mr Leslie Grainger, the science member on the UK's National Coal Board, which will be the operating agent for each of the five projects. The hope is that the programme will cut the costs of heat for industry and for generating electricity by making a more efficient use of coal.

● An agreement concluded earlier this month will effectively make the UK's Chemical Society owners in the British Isles of all the abstract data produced by the American Chemical Society's Chemical Abstracts Service (CAS). In exchange, the CAS will receive payment in kind in the shape of the specialised expertise developed by the UK Chemical Information Service (UKCIS), a part of the Chemical Society based at the University of Nottingham.

Chemical abstract services are quite big business nowadays, with a total of 5 million chemical compounds known at present and 5,000 new ones being discovered every week. Chemists report their work in fully 14,000 journals.

Even keeping up with the abstracts is itself a considerable chore for many chemists, and the cynics say that it is sometimes quicker to do a simple experiment (to determine a melting point, for example) than to look up

Round Britain

the information in *Chemical Abstracts* (the present five-year index of which runs to 68 volumes!).

Although the CAS spends some \$25 million a year putting the abstracting material on to a computer and producing *Chemical Abstracts*, it has paid little attention to the problem of how to manipulate further such a vast data base. In the USA, information centres run by bodies like Battelle and Lockheed reprocess the information for their own particular purposes.

The UKCIS, however, has made a point of developing personalised services by making available to individual chemists a current-awareness service which caters for their individual interests. It also, for example, publishes subject-oriented bulletins, 'Macroprofiles'.

At present the UKCIS markets and distributes *Chemical Abstracts*, and some other publications and services of the CAS, in the British Isles. As its contribution to the CAS it also produces the 5% or so of all CAS abstracts and index entries which originate in papers published in the UK.

The annual budget of the UKCIS is, however, small beer by comparison with its American counterpart. Its non-capital expenditure at present is £1.2 million a year. But, like the CAS, its aim is to break even.

The UKCIS can obviously widen its horizons quite considerably now that it is much more than simply an agent for the CAS. In particular, it is actively considering, together with the Department of the Environment, the setting up of a data bank on environmentally significant chemicals. Called DESCNET, it would allow biological data on chemical compounds to be stored with other information about them and "should assist in the freer flow of information concerning potential environmental hazards".

● The Post Office exhibited much of its current work in telecommunications research and development last week when the Queen opened the new £11 millions Post Office Research Centre at Martlesham Heath in Suffolk. But most of it was unexpectedly overshadowed by the news that the Post Office is to place major contracts with the telecommunications industry for the development of a computer-controlled switching system code-named System X.

The new system, which will use the latest microelectronics technology, will lead to a modern telecommunications network in Britain capable of handling the wide range of services expected to be in demand by the 1980s, including visual transmission and high speed data print-outs. The development of System X, which is expected to take from five to seven years and cost up to £100 millions, is reportedly the biggest single item of expenditure of the Post Office's annual research bill.

The joint development programme will involve about a dozen contracts with such firms as Plessey, GEC, STC and Pye TMC, the final details of which have yet to be drafted. Advanced as System X is compared to existing telephone switching systems, it is unlikely to supercede current Post Office research work in this field. Both the new waveguide system, which is capable of handling up to half-a-million transmissions simultaneously, and developments in the field of fibre optics will provide important contributions to it. Indeed, it is because System X is based on a series of sub-systems that it offers the Post Office a good degree of flexibility as the development matures.

● A research team at the Harwell Atomic Energy Research Establishment has developed a miniature, nuclear-powered battery which may, quite literally, provide a boost to sufferers with certain types of heart disease. Designed to fit into a small "pacemaker", the new battery works on the thermocouple principle, and because the power output of its plutonium 238 heat source falls off by only about 1% a year it could have an implanted lifetime of 10 or even 20 years. By contrast, conventional chemical pacemaker batteries rarely operate for more than three years before they need to be replaced surgically. The Department of Health and Social Security has ordered 100 pacemakers from British Nuclear Fuels Limited (BNFL) for chemical trials, and the device, now costing about £2,000, could be worth about £10 millions to BNFL.

correspondence

Food for thought

SIR,—Dr Magnus Pyke in his article "All the food that's fit to eat" (October 23) notes that the "Society of Public Analysts, now the Society for Analytical Chemistry", was established in the same year as the Sale of Food and Drugs Act 1875. In fact, the first president and council of the new society were elected in December 1874, and were instrumental in influencing certain amendments to the 1875 Act, which was first introduced to the House in February 1875. The society was known as the Society for Analytical Chemistry from 1954 to December 1974, and from January 1975 has continued its activities as the Analytical Division of the Chemical Society.

Yours faithfully,
F. D. GRIMMER

*The Chemical Society,
London W1, UK*

Aiding Vietnam

SIR,—In his report "Vietnamese Journey" (November 6) Arthur Galston put forward various suggestions for an American aid programme to help Vietnamese scientists. These included sending scientific literature, equipment and visiting scientists to Vietnam.

On the British scene two of these recommendations have been in operation for several years. The Medical Aid Committee for Vietnam has sent considerable quantities of current medical literature to doctors in Vietnam since 1967, and in 1973 began sending scientific literature to Professor Hieu. Many British scientific institutes have assisted us in this work by making donations, including the Biochemical Society, the Institute of Physics, the Royal Statistical Society and the Nutrition Society. Similarly many libraries, including the Library Association, now contact us when they have material which is surplus to their requirements and which we consider suitable for dispatch. All literature is sent free of charge, courtesy of the Soviet Red Cross.

Regarding scientific equipment and chemicals, the Trade Union Committee for Reconstruction in Vietnam has sent material worth some thousands of pounds to Professor Hieu for use in both the north and south of Vietnam.

On a more institutional level, Professor N. W. Pirie last year visited North Vietnam on behalf of the Royal Society and this year the British Government made a donation of scientific, medical and English literature to three institutes in Hanoi.

Several scientific institutes in the UK have expressed a desire to continue to supply current literature to Vietnam. This could easily be arranged, either through the British Council or the Ministry of Overseas Development, and would not require excessive funds. Our committee put forward these suggestions to both the British Council and the Ministry several months ago and we are waiting for their decision.

Perhaps now that more British scientists are aware of the problems facing their Vietnamese colleagues as a result of Professor Arthur Galston's article, there will be greater developments in the British aid programme to Vietnam.

Yours faithfully,
A. HAY

*Medical Aid Committee for Vietnam,
London WC2, UK*

Journal guidelines

SIR,—It is good that you saw the purpose of the document on "Authors, Editors and Referees" issued by the Scientific Information Committee of the Royal Society—that is, to initiate well informed discussion on this fundamental element of the communication system of science, and to bring into the open the actual procedures that are used (November 6). Indeed, it is only by showing that these procedures are honest and just, giving a fair weight to the interests of all parties concerned and to the needs of the scientific community at large, that we can justify our resistance to the demand to disclose the names of referees to authors, and similar populist follies.

You are also correct in pointing out that the proposed 'guidelines' are not appropriate for your own journal. We are all happy with the thought that *Nature* is almost unique in the world of science. And I would not apply precisely this code to a review journal such as *Reports on Progress in Physics*. But the question whether editors of conventional primary research journals are well advised to deviate very far from the suggested code is worth open

and collective debate and should not be confined to laboratory coffee clubs or to the committee rooms and editorial boards of individual learned societies.

May I suggest that by discussing this document in an editorial, quoting only points with which you disagree, you underestimate the intelligence and good sense of your readers, who might like to decide for themselves whether it had some merit in general. Since the whole 'code' is scarcely longer than this letter, why not publish it in full for wider discussion?

Yours faithfully,
JOHN ZIMAN

*University of
Bristol, UK*

Here it is:

(i) Every paper submitted to the journal for publication should be refereed.

(ii) Referees are appointed by the Editor, and report to him.

(iii) The name of any Referee may only be disclosed to the Author by permission of or at the request of the Referee, with the Editor's agreement.

(iv) The Referee may not disclose the contents of a paper submitted to him, nor make use of these for his own scientific work before publication, without explicit permission of the Author, to whom the name of the Referee must then be disclosed.

(v) The Referee's report should contain a definite recommendation concerning publication based upon a reasoned judgement of the general form and scientific contribution of the paper as a whole. He may recommend acceptance outright or subject to revision of certain sections along specified lines, or may propose that the paper be substantially rewritten to improve the presentation or to strengthen the scientific argument. The substance of all such critical comments should be communicated to the Author for action, to the eventual satisfaction of the Referee. But failure to accept the Referee's view on all minor points of criticism should not be a bar to publication.

(vi) No paper should be rejected on the adverse report of a single Referee.

(vii) In the case of conflicting opinions, the Editor may appoint further Referees or an Adjudicator who may read all communications between the Author and Referees (whose names need not be disclosed to him).

(viii) A definite procedure should be established for Editorial decision within a stated period.

We disagree with i, vi, and viii (but see our statement of editorial procedures on page 1 of the November 6 issue) and would like to hear what others think.

Ed.

news and views

Successional patterns and indices of diversity

from Robert M. May

ONE way to quantify Robert Louis Stevenson's belief that "the world is so full of a number of things" is simply to count how many things there are. The result gives the 'species richness', the number of plant or animal species observed to be present in a given community.

But usually one wants to know more than this. If 20 species are present, are they roughly equally abundant, or is one common and the other 19 rare? A full answer to this question involves finding the distribution function which describes the relative abundance of all the species in the community. On the other hand, one may wish to settle for less, and be content with some statistical moment of the distribution, some single number, which characterises the diversity of community. A large (and often muddled) amount of the ecological literature is devoted to the search for such 'diversity indices'. A lucid account of some mathematical aspects of these questions is in the monograph just published by Pielou, *Ecological Diversity* (Wiley; 1975).

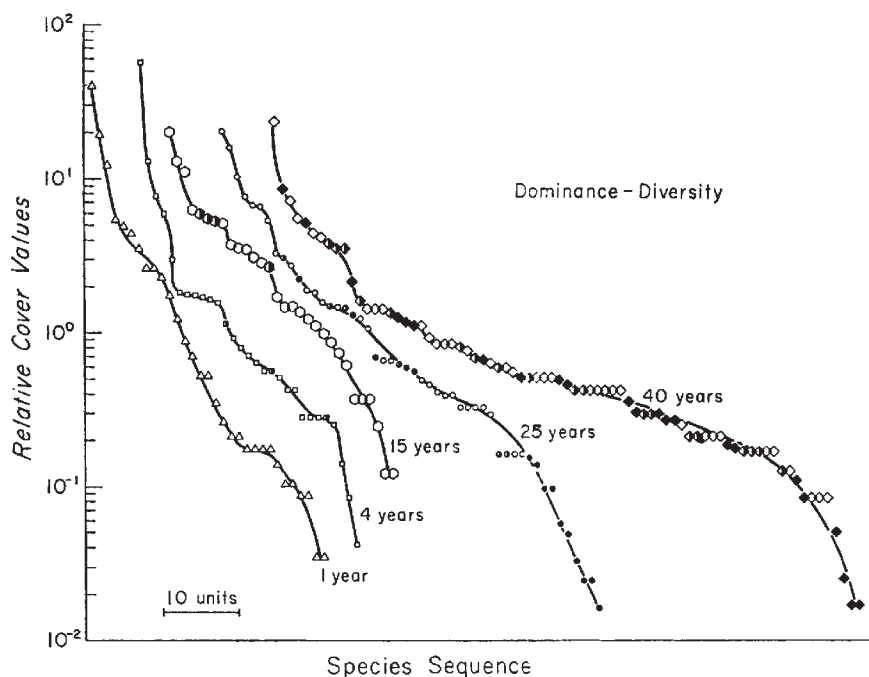
Field studies of the relative abundance of species in a variety of plant and animal communities have revealed interesting patterns, many of which can be plausibly explained on theoretical grounds. In the early stages of succession, plant communities will tend to consist of a handful of weedy, pioneer, r-selected species that happened to get there first. The species arriving first will tend to grow rapidly, pre-empting a fraction (k say) of the available space or other governing resource before the arrival of the next species, which in turn will tend to pre-empt a similar fraction of the remaining space before the arrival of the third, and so on. The consequent pattern will be, very roughly, a geometric series in relative abundance. As succession proceeds, things become more complicated, and a multitude of ecological dimensions are likely to be relevant to the ultimate composition of the community. That is, in later successional stages the relative abundances of the various species are governed by the interplay of many more-or-less independent factors. It is in the nature of the equations of popu-

lation dynamics that these several factors should compound multiplicatively, and the statistical Central Limit Theorem applied to such a product of factors implies a lognormal distribution (that is, a bell-shaped gaussian distribution in the logarithms of the species' abundances). Once many species are involved, a lognormal distribution gives a much more even pattern of species relative abundance than does a geometric series distribution: the lognormal is a socialist society compared with the feudal hierarchy of the geometric series.

These notions are in accord with the data obtained from several studies of plant succession at various locations in the temperate zone (see, for example, Whittaker, *Communities and Ecosystems*, ch. 4, second ed, Macmillan, New York; 1975). The patterns show up with particular clarity in the recent study by Bazzaz (*Ecology*, **56**, 485-488; 1975) of old fields in southern Illinois,

at successional stages varying from 1 to 40 years. When the logarithm of species relative abundance is plotted against the rank of the species in an abundance hierarchy, early successional stages show the straight line relationship characteristic of a geometric series, while later stages show a progressively more S-shaped curve, with a preponderance of "middle class" species, characteristic of a lognormal distribution. The figure, taken from Bazzaz's paper, makes this plain.

Something akin to a reversal of these successional patterns takes place when mature communities become polluted. This has been shown particularly for streams and lakes subject to "enrichment" by waste heat, sewage, and other materials. The most detailed studies are those of Patrick and coworkers on the changes in patterns of species relative abundance in diatom communities. The equilibrium diatom community essentially always



Patterns of species relative abundance in old fields of five different stages of abandonment in southern Illinois. The patterns are expressed as the percentage that a given species contributes to the total area covered by all species in a community, plotted against the species' rank, ordered from most to least abundant. Symbols are open for herbs, half-open for shrubs, and closed for trees (from Fig. 2 of Bazzaz, *Ecology*, **56**, 487; 1975).

shows the classic lognormal distribution; when polluted, the community typically shows a pattern in which a few species become exceptionally common, with a hierarchical geometric series distribution (possibly caused by an early successional type of r-selection for success in handling the all-important new factor). Closer analysis may, or may not, reveal a lognormal distribution of relatively rare species hanging on down in the tail of the distribution.

These connections between patterns of relative abundance and degree of pollution have sparked off interest in the possibility of using some index of diversity to measure how much an ecosystem has been perturbed by human activity. One such attempt, using a diversity index as a phenomenological measure of gross toxicity in San Francisco Bay, is due to Anderson *et al.* (*Proceedings of 5th International Conference on Water Pollution Research*, Pergamon; 1971). Clearly, an engineering-style index of

this kind would simplify tasks of environmental management. But Patrick (private communication) emphasises that, for her diatom communities in polluted streams, any such single number will be dominated by the geometric distribution of the common species, whereas the time scale for recovery of the pristine ecosystem (and, indeed, whether it can ever recover) depends on the lognormal tail of species which are uncommon in the polluted community, and whose presence will not show up in any overall diversity index. This amounts to a cogent argument (similar to that advanced by Williamson, in *The Mathematical Theory of Biological Populations*, Academic Press, New York, 1973) for describing the community by its full distribution of species relative abundance, and not trying to condense information into a single diversity index, which may mislead. The whole subject has practical importance for environmental impact studies, and deserves more attention than it has received to date. □

Earthquake prediction in China

from David Davies

IN March 1966 two severe earthquakes struck Hopeh Province, China; the scene of the disaster (relatively close to Peking) was visited by Chou En-Lai in his capacity as Prime Minister. Fortuitously the early part of 1966 also saw the start of the Great Proletarian Cultural Revolution. Earthquakes in China had caused great loss of life throughout history (nearly a million people died from one in 1556); what more logical project for a rebuilt people-oriented science programme than to attempt to predict earthquakes. And what more logical approach to this problem than to involve the general public as intensively as possible.

Nearly ten years later the fruits of this remarkable enterprise are beginning to become visible to Western eyes. In the November issue of *Eos*, the Transactions of the American Geophysical Union, is presented an extensive report of the American Seismology Delegation of October 1974 to China. Coincidentally, travellers' tales from more recent visitors to China begin to confirm the successful prediction of a very large earthquake on February 4, 1975—a prediction that may well have saved many thousand lives.

China does not fit simply into the scheme of plate tectonics. Scattered continental seismicity did not lend itself to the drawing of unambiguous plate boundaries, and it has taken several years to integrate what is known about surface geology, broad-

scale lineations, earthquake distributions and their focal mechanisms into a reasonable tectonic synthesis. The work of Molnar and Tapponnier (*Science*, **189**, 419–426; 1975) suggests, for instance, that much of the seismicity in China can be considered as a result of the taking up of the convergence of the Indian subcontinent on Eurasia at a rate of 5 cm per year, with the concomitant necessity for parts of Eurasia to slide laterally out of the way. One of China's enormous assets is an earthquake record of sorts which goes back 3,000 years. North China in particular, has a record which the delegation called 'remarkably complete' for large events since 466 BC, and homogeneous for even small (magnitude 5) events since 1484 AD. The group noted that comparable dates for southern California would be 1850 and 1930. And yet even with this quality of reporting the patterns still remain complex, and as a result any programme of vigilance has to cover a substantial area of the country.

Ten thousand professionals participate in the Chinese earthquake programme, ten times the number in the United States, and there is a band of many thousand amateurs. Activity is spread widely over the country and the instrumental content of the programme is equally broadly based. Work is being conducted on all types of premonitory symptoms studied in the West—seismic, geodetic, electromagnetic, geochemical, magnetotelluric; and in addition there

is considerable interest in acoustic precursors (earthquake sounds) and the unusual behaviour of animals. These last two phenomena have had a very shadowy history in the West—there is practically no scientist concerned about his professional reputation who would have ventured to express enthusiasm for them. But in China, where everyone is encouraged to report unusual events, there is a much greater interest in such possibilities.

Despite the encouragement of central government to pursue science for the people, it took five years from 1966 before the nationwide programme began in earnest. The turmoil at the Academy of Sciences during the cultural revolution must have been one major factor, as there was obviously a lack of steady central administration. Another factor must undoubtedly have been inadequate practical experience amongst geophysicists; the legendary ability of the Chinese has been to solve theoretical problems. Since 1971, however, the programme has developed enough confidence to issue at least eleven predictions which have led to extensive evacuations of threatened regions. Precise details both of the success rate and of the total data sets used have not been released, but there is no doubt at all that there have been major successes.

Ironically three months after the delegation had left China the most spectacular prediction came, for which, as yet, we have only the reports of more recent visitors. It seems that Liaoning Province had for several years been viewed as a possible target for a major earthquake as a result of migration of seismicity, and in the months before the quake, tilting occurred and there were observations of changes in well levels, telluric currents and animal behaviour. Then immediately before the magnitude 7.3 event, foreshocks gave sufficient warning for mass evacuations. There was enormous damage—a town of 100,000 is being completely rebuilt—but few fatalities.

Satisfaction at this major success must be tempered with some concern over the way in which the work will now advance. One of the disturbing themes running through the American delegation's report is an apparent weakness in more basic fields of research. The pendulum seems to have swung so far from theory that it is questionable whether there is adequate work going into the fundamental understanding of the processes that are being observed. Visitors speak of a far too ready acceptance of the work of any and everybody without the profound criticism that advances science most rapidly.

The successes of the Chinese pro-

gramme come at a particularly crucial stage in the development of the United States project under the auspices of the Geological Survey. Recently an increasing amount of attention has been paid to the public policy aspects of prediction with, at least in some people's opinion, a concomitant build up in the public expectation that scientists are going to be able to deliver the goods in the very near future. Yet many seismologists privately entertain serious doubts that the premonitory symptoms are anything like unambiguous enough to warrant going public for some years to come. Three weeks ago Dr V. E. McKelvey, Director of the Geological Survey, was saying at a conference on earthquake warning and response, 'we are now entering an age when scientific instruments are detecting geophysical signals that can be interpreted to forecast earthquake occurrence'. Many would have preferred him to have put this age five or ten years in the future while some very difficult work is being done. Unfortunately, although the Chinese results are very exciting indeed, they may give the false impression that the United States is in for a similar pay off. This is rather unlikely. Foreshocks, the means by which the Chinese pinned down the time of the quake so accurately, are a relatively rare occurrence before major events, and other techniques used such as telluric currents, migration of seismicity and radon levels in ground water have yet to show their usefulness in the United States.

One thing is indisputable, however. The Chinese work will have acted as a shot in the arm to seismologists in all other countries engaged in prediction research. And by all accounts there is more excitement still to come. □

Messengers in minicells

from M. R. Blundell

THE first experiments demonstrating the existence of messenger RNA in bacteria were published in 1961. One of the criteria for identification of mRNA was its short lifetime, which permits a rapid halt in synthesis of a specific protein, but our knowledge of the processes responsible for inactivation and degradation of mRNA remains very incomplete fourteen years later.

We know that degradation proceeds from the 5' to the 3' end of mRNA (Morikawa and Imamoto, *Nature*, **223**, 37; 1969) but it is clear that the 5'-exonuclease activity called ribonuclease V (Kuwano, *et al.*, *J. molec. Biol.*, **51**, 75; 1970) cannot be responsible alone for mRNA degradation (Imamoto and Schlessinger, *Molec. gen. Genet.*, **135**, 29; 1974).

What was regarded as merely a scavenging process has taken on new significance with the discovery that individual messages in the same *Escherichia coli* cell can have different decay rates with half-lives ranging from 40 s upwards at 37 °C (Marrs and Yanofsky, *Nature new Biol.*, **234**, 168; 1971; Blundell *et al.*, *Nature new Biol.*, **238**, 46; 1972; Craig, *et al.*, *J. molec. Biol.*, **71**, 701; 1972, and others). Even messages in the same polycistronic mRNA can have quite different decay rates.

Hirashima, Childs and Inouye (*J. molec. Biol.*, **79**, 373; 1973) discovered a message with a particularly long functional half-life, 11.5 min at 37 °C, which codes for a low molecular weight lipoprotein of the outer membrane of *E. coli*. Both *et al.* (*J. molec. Biol.*, **67**, 199; 1972) presented measurements of the secretion of an extracellular protease by *Bacillus amyloliquefaciens* which could be interpreted in terms of a mRNA stable for up to 80 min at 30 °C, but subsequently, they concluded (Glenn *et al.*, *J. molec. Biol.*, **73**, 221; 1973) that this message was not especially stable, but was instead made in very large quantities. In hybridisation experiments, however, Brown and Coleman (*J. molec. Biol.*, **96**, 345; 1975) could not find such quantities of mRNA, or any especially stable mRNA in cultures of *B. amyloliquefaciens*.

Now Levy (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 2900; 1975) reports the presence of a small quantity of very long lived mRNA in *E. coli* minicells, structures about one tenth the size of whole cells which are made in abnormal cell divisions of an *E. coli* mutant and lack the *E. coli* chromosome. Plasmid DNA that segregates into minicells is transcribed and translated, but plasmid-less minicells do not make DNA or RNA. Levy observes, however, that ³⁵S-methionine is incorporated by freshly isolated minicells into hot trichloroacetic acid precipitable material at 0.2 to 0.5% of the rate in whole cells. The incorporation is sensitive to chloramphenicol, so it is presumably ribosomal polypeptide synthesis. Protein synthesis decays during incubation at 37 °C with a half-life of about 80 min, and is unaffected by rifamycin. The major products of protein synthesis in plasmid-free minicells are four polypeptides of the outer cell membrane, one of these being the lipoprotein coded by the long lived mRNA studied by Hirashima *et al.*

We can, therefore ask, as does Levy, why the messages for membrane proteins should be so stable in minicells. Minicells containing an R factor continue to make short lived mRNA which codes for other proteins, so mRNA can be degraded in minicells, but no comparison of the decay rates

of R factor messages in whole cells and minicells is presented. One possibility, since minicells form from the poles of cells, is that the cell extremities, and therefore minicells, are relatively deficient in specific nuclease(s).

We may also ask why membrane proteins should have abnormally stable message. To investigate this question Hirashima, Wang and Inouye are now developing a cell-free translation system which synthesises the lipoprotein when purified mRNA is added. □

Unexpected proviruses in chronic infection

from Robin Weiss

RECENT reports by Zhdanov (*Nature*, **256**, 471; 1975) and by Simpson and Iinuma (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 3230; 1975) promise to open up a new area of genetic exchange between RNA viruses and their hosts. These authors claim that some animal RNA viruses that conventionally replicate by means of RNA intermediates can under conditions of chronic infection form DNA "proviruses".

Simpson and Iinuma have studied a temperature sensitive mutant of respiratory syncytial (RS) virus in bovine embryo kidney (BEK) cells infected and maintained at the non-permissive temperature of 39 °C. These BEK/RS cells did not release RS virus or synthesise RS viral antigens detectable by immunofluorescence. However, even late passage cultures yielded infectious virus when co-cultivated with uninfected cells or when treated with agents such as halogenated pyrimidines. These findings led the authors to look for DNA proviruses of RS virus using transfection techniques as described by Hill and Hillova (*Nature new Biol.*, **231**, 261; 1971) for the provirus of Rous sarcoma virus. When HEP-2 cells were treated with DNA extracted from the BEK/RS cell line infectious RS virus was recovered. A clonal analysis of the progeny RS virus indicated that some clones retained the special properties of the virus that initiated the chronic infection—temperature-sensitive replication and heat-resistant virions—whereas other clones had lost one or both of these properties and more closely resembled wild-type RS virus. Simpson and Iinuma further showed that a chronically-infected cell line of HEP-2 cells, survivors of an infection with wild-type RS virus, similarly contained infectious DNA for RS virus.

Zhdanov's analysis is less detailed but wider ranging, and illustrates the same phenomenon of DNA provirus formation in cultures chronically in-

fects with RNA viruses. Persistent infections were established with measles virus in chick embryo cells, Sindbis virus in murine L cells and tick-borne encephalitis virus in HEP-2 cells. Using tritiated viral RNA, Zhdanov showed by hybridisation *Cot* analysis that the chronically infected cells in each of the three systems contained sequences homologous to the viral RNA, whereas the uninfected control cells did not. With the chronically infected HEP-2 cells he was also able to recover infectious tick-borne encephalitis virus by DNA transfection.

The formation of proviruses demands transcription of RNA to DNA and both groups found 'reverse transcriptase' activity associated with the chronically infected cells. Zhdanov assumes that the persistently infecting virus interacts with latent oncornaviruses present in the cultures. It will be interesting to determine whether the presence of oncornavirus reverse transcriptase is necessary for provirus formation, and whether the provirus is a prerequisite to or a consequence of the development of such persistent infections. What is remarkable is that not only are some DNA sequences transcribed, but whole, transfectable proviruses are formed, suggesting a process of orderly, complete reverse transcription. Simpson and Linuma also comment in their discussion that plaque-purified RS virus recovered from DNA transfection carries reverse transcriptase activity and may represent recombinant virus species. One wonders, therefore, whether other lytic viruses which replicate by way of a DNA provirus, such as visna virus and avian reticuloendotheliosis virus, might have evolved in a similar way and might possess alternative modes of replication.

These studies raise interesting questions not only for molecular biologists but also for students of persistent infections and 'slow' virus diseases. Zhdanov presents evidence for measles virus DNA in tissues of patients suffering from systemic lupus erythematosus. No doubt the hunt is on for DNA copies in other 'viral' autoimmune diseases. □

Martian canals after Mariner 9

from David W. Hughes

EVER since 1877 when Schiaparelli drew attention to the existence of canals on Mars they have been the subject of heated controversy and, at times, distinct embarrassment to planetary astronomers. Mariner 9, the spacecraft that went into orbit around

Mars in 1971, achieved a complete mapping of the surface down to a resolution of 1 km, so now the question, "where are the canals?" should be answerable. Sagan and Fox of the Laboratory for Planetary Studies, Cornell apply themselves to this question in a recent issue of *Icarus* (25, 602; 1975).

Many suggestions have been put forward to explain the observations, observations which cannot be discounted out of hand, because, even though the features are of intrinsically low contrast and small angular size usually observed under poor seeing conditions, many maps of tolerable mutual consistency have been produced by different astronomers.

Canals have been thought to be waterways or the vegetation along the banks of waterways constructed by an advanced civilisation. Natural valleys, surface cracks or ridges produced by a range of geological processes were another suggestion. Accidental alignments of surface features (for example chains of large craters) was a third. They may also be rectilinear sand dunes or dark rays emanating from craters, or simply albedo features, regions of differing reflectivity that are periodically covered and uncovered by windblown dust.

Sagan and Fox compare the Mariner 9 results with the canal cartography of Slipher (*Mars*, Sky Publishing Co., Cambridge, Mass; 1962). A striking correlation was found between the canal Agathodaemon and the great rift valley Valles Marineris but as the latter is a low albedo feature 5,000 km long and 100 km wide it is only to be expected that this is easily resolvable by ground-based observers. Thoth-Nepenthes is also easily discernible on both maps as is the ridge Ceraunius.

Returning to the hypotheses concerning canal origin, Sagan and Fox conclude that the resolution and surface cover of Mariner 9 would have detected a civilisation like Earth's if it had been present on Mars. Correspondences do occur between classical canals and valleys or ridges, Agathodaemon-Valles Marineris has been mentioned and Styx-Phlegra Montes and Ceraunius are other examples. Psychophysiological alignments of disconnected fine detail only seem probable in the case of the canal Oxia which seems to be the chain of craters Trouvelot, Rutherford, Becquerel, Curie and Sklodowska and an unnamed canal which goes through craters Galle, Wirtz, Helmholtz and Lohse. Very few crater rays were seen by Mariner 9 and sand dune fields do not seem to be sufficiently extensive to be observed from Earth. A few canals notably Cerberus and Thoth-Nepenthes do correspond to dark features on the

Martian surface, these features being unconnected to topography. They might, however, be regions where fine bright dust does not settle or regions where the ground is dark due to surface roughness or some geochemical reason.

While a few canals do correspond to topographic and albedo features on Mars, Sagan and Fox conclude that the large majority of canals do not. Conversely there are many real surface features, for example large shield volcanoes like Olympus Mons, Arsia Mons and Pavonis Mons, where no canals were marked on the maps.

Sagan and Fox discount the canal observations and relegate them to monuments to the imprecision of the human eye labouring under difficult seeing conditions. But does the story really end here. Probably so, but the consistency of the diverse maps leaves a lingering unease. Maybe the facts that Mariner 9 could not distinguish between features which had less than 5% albedo contrast, that Mars was only observed for half a Martian year, or the violent dust storms that preceded Mariner 9's visit caused the real truth to be hidden. □

Parity determination with polarised deuterons

from P. E. Hodgson

A NEW technique for determining the parity of nuclear states has been developed recently by Kuehner *et al.* (*Phys. Rev. Lett.* 35, 423; 1975) at McMaster University, Hamilton, Ontario. The method depends on the properties of the coupling between the orbital and spin angular momentum vectors in the initial and final states. In certain conditions the yield of particles from a reaction at 0° (or 180°) on an even-even target is zero for states of natural parity $(-)^J$, so a single measurement determines the parity if the spin J of the state is known.

The conditions for this to hold are that the magnetic quantum numbers m are zero in the initial and final states. Since these are the z -components of angular momenta (taking the beam direction as the axis of quantisation) this may be ensured by using a polarised incident beam with $m=0$ and detecting the products of the reaction along the beam axis, that is at 0° or 180°. Nucleons have $m=\pm\frac{1}{2}$, and so cannot be used, but deuterons having spin one can be in the $m=0$ or ± 1 states, and it is possible to polarise the beams.

Although this method is simple in principle, it is not simple to carry out in practice. It is difficult to produce polarised beams, and existing techniques can-

not yet give 100% polarisation. It is therefore necessary to compare the results of measurements with unpolarised and partially polarised beams. It is also difficult to make measurements very near to 0° , but a subsidiary study showed that in practice the intensities of the forbidden reactions are very low below 2° , and so measurements made at this angle are sufficiently good.

Kuehner *et al.* applied this technique to the reaction $^{12}\text{C}(\text{d},\alpha)^{10}\text{B}$, and some of their results are shown in the figure. The upper spectrum was obtained with an unpolarised beam and the lower with a partially polarised beam. It is immediately evident that the relative intensities are different, and the actual ratios are also displayed. The intensities of the states with parity $(-)^J$ are most strongly reduced, though not to zero, because of the residual $m=\pm 1$ components in the parti-

ally polarised beam. A subsidiary calculation shows that these states should be reduced by the ratio indicated by the dashed line in the figure.

These ratios therefore give immediately the parity of the final state of ^{10}B : those with ratios on the dashed line have parity $(-)^J$ and those with ratios above the line have parity $(-)^{J+1}$. Since the spins are usually known, this gives the required parities. There may be some cases where the parity is known and the spin from other measurements must have either of two consecutive values; in this case the method determines the spin.

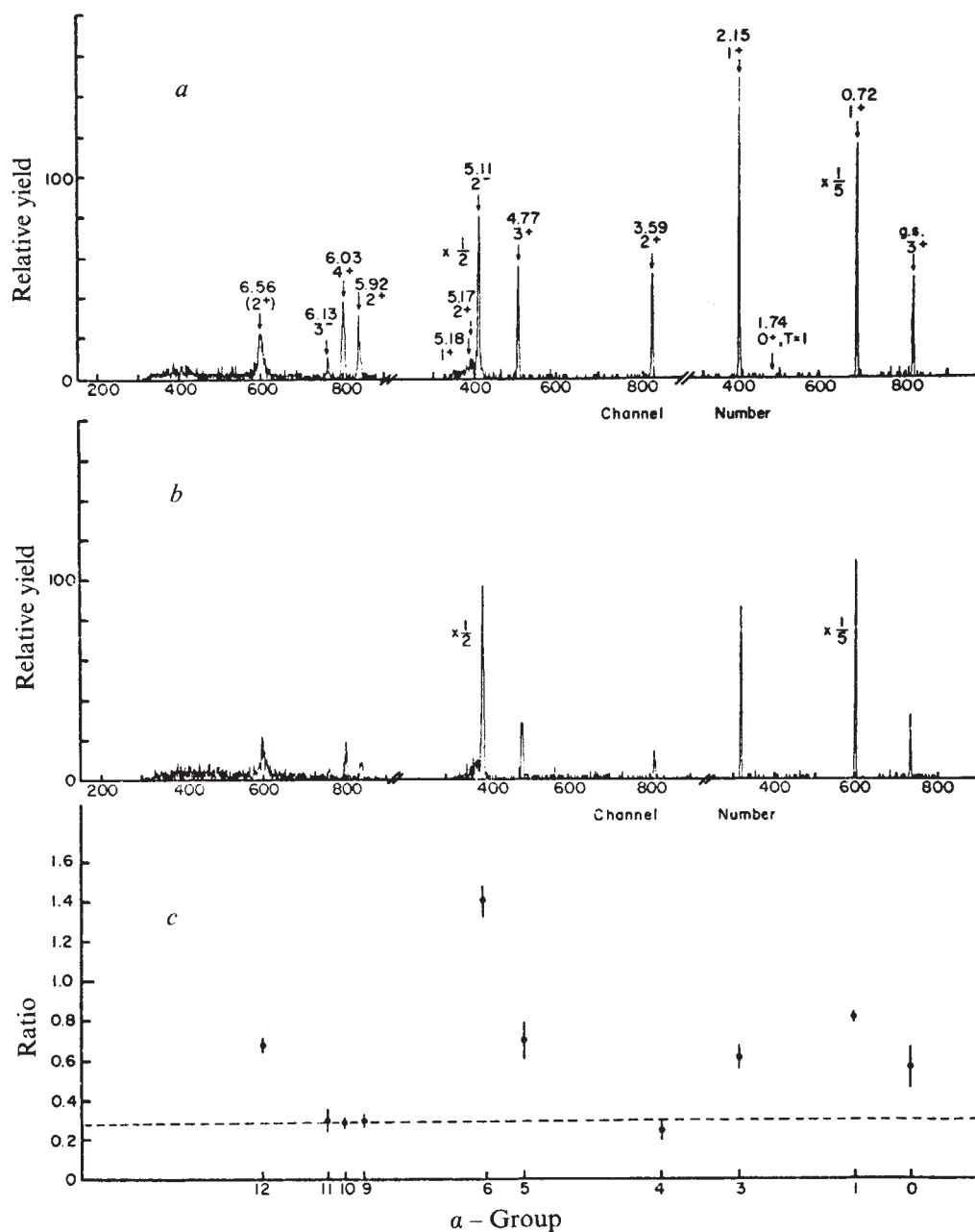
An example of this latter situation is provided by the state of ^{10}B at 6.56 MeV. Previous measurements had indicated 2^+ , though 3^+ was also a possibility. The measurements of Kuehner *et al.* show that the spin must be odd, and thus the assignment 3^+ is confirmed.

This new technique should prove a powerful way of determining the parities or spins of nuclear states. If both the spin and the parity are known, it can be also used to determine the tensor polarisation of a beam of spin-one particles; this could be particularly useful for projectiles like ^6Li for which the usual double-scattering method is difficult. $\square$

Seed dispersal

from Peter D. Moore

SEED production, seed germination and seedling establishment are, for most plant species, critical stages in the maintenance of population levels. The quantity of seed produced and the energy reserve of each individual seed vary from one species to another;



Spectra of alpha-particles from the reaction $^{12}\text{C}(\text{d},\alpha)^{10}\text{B}$ at 2° to the incident beam for an unpolarised (a) and for a partially polarised beam. (b) Also shown (c) is the ratio of the intensities for the two cases; this has the value shown by the dashed line for states with parity $(-)^J$ and a higher value with states with parity $(-)^{J+1}$.

Harper, *et al.* (*A. Rev. ecol. Syst.*, **1**, 327; 1970) pointed out that r-selected species often produce large quantities of small seeds whereas K-selected ones often have a small number of large seeds. Survival of seeds depends upon the avoidance of predation, and numerous mechanisms have evolved by which such avoidance may be effected, for example by toxicity or by rapid and widespread dispersal (Janzen, *Evolution*, **23**, 1; 1969). The scattering of seeds besides reducing the risks of predation, also assists the avoidance of intra-specific competition among seedlings and permits the spread of the species to new localities, thus aiding the exchange of genetic material.

A recent study of seed production and dispersal in the genus *Viola* by Beattie and Lyons (*Am. J. Bot.*, **62**, 714; 1975), illustrates the complex results produced by these interacting selective pressures. Initial studies of seed dispersal in the genus led to the classification of *Viola* species into two major groups, one myrmecochorous (dispersed by ants) and the other diplochorous (explosively ejected from the capsule and subsequently further transported by ants). Diplochorous types have many morphological features associated with projectile dispersal, such as an erect, tall peduncle to the capsule and a relatively small caruncle on the seed when compared with the myrmecochorous types. The explosive ejection of these seeds often results in their being thrown 1-5 m from the parent plant.

The possession of a caruncle by the diplochorous types suggests that there is a subsequent stage in dispersal when the caruncle may serve as an attractant and a food reserve for the transporting agent. If this were not so, then selection would presumably have resulted in the loss of the caruncle in these species in the interest of aerodynamics. To examine this, Beattie and Lyons placed different types of *Viola* seed in areas of woodland in West Virginia and also in prairie in Illinois where ant nests were abundant. The diplochorous seeds were collected by ants when fresh whether they were placed in groups or scattered as individuals, but the rate of collection was considerably lower if seeds from the previous season were used. Evidently their attractiveness to ants is adequate while they are fresh, but deteriorates with age.

In the case of the myrmecochorous types, only old seed was available. These were placed in groups to simulate the natural situation when such seeds would remain in their capsules. It was found that the rate of disappearance was as rapid for these old seeds as in the case of the fresh diplochorous seeds. The myrmecochorous types evidently have mechanisms of

attraction which are more persistent than those of the diplochorous types.

The myrmecochorous types have evolved a dispersal strategy in which they combine tough seed coats, easily accessible energy-rich seeds located in groups, with a frequent recurrent production of new seeds. The danger of such a strategy lies in the close grouping of the seeds, which exposes them to predation, and this is overcome by the development of mutualistic relationships with specific ant species. These ants collect the seeds regularly from predictable localities and carry them to their nests where they feed upon the caruncle, leaving the embryo unharmed and able to germinate. Predation avoidance is thus dependent upon collection and dispersal by ants. The heavy seeds and large, energy-rich caruncles of these species assist in the strategy, which can be considered as an essentially K-selected one.

The diplochorous mechanism provides an alternative solution to the dangers of seed predation. Local concentrations of seeds are avoided by the explosive dispersal mechanism, but the retention of the caruncle ensures that the seeds will be found and further dispersed by ants. In the experiments, almost all fresh diplochorous seeds had been found and removed within three days. Beattie and Lyons suggest that full dependence on ant dispersal is only possible in situations where an ant species with the appropriate behaviour pattern is abundant. In sites with depauperate ant faunas, the diplochorous strategy is more reliable. This may account for the geographical restriction of the myrmecochorous *Viola* species to those regions of Eurasia where the mound-building, scent-trail laying ant species *Formica rufa* is found. □

Fishing for complement

from Robin Hesketh

THE study of the movement of protein complexes in the plane of the fluid membrane has kept a good many immunologists off the streets since the demonstration in 1971 by Taylor, Duffus, Raff and de Petris of the redistribution of lymphocyte surface immunoglobulin molecules after their interaction with antibody. It now seems that a venerable method for measuring the binding (fixing) of complement (C) by antigen-antibody complexes may assist in defining the necessary environment for membrane receptors to participate in some immunological reactions.

It is now well known that the fluidity

of membranes can be changed by varying the amount of cholesterol present, and Humphries and McConnell (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 2483; 1975) have used this fact to correlate the lateral mobility of a membrane antigen with its C-fixing ability.

As a model membrane they used a lecithin bilayer containing cholesterol into which the monovalent lipid antigen cardiolipin had been introduced. Complement binding by this system in the presence of human syphilitic serum, which contains antibodies against cardiolipin, provides one of the simplest models for immune cytotoxic attack.

This system of lipid suspensions, later christened liposomes, was first developed by Osler and Knipp (*J. Immun.*, **78**, 19; 1957) in a search for a precise method for estimating the cardiolipin-specific Wasserman antibody in human syphilitic serum. They showed that the low antigenic activity of suspensions of monovalent cardiolipin was greatly enhanced by the addition of lecithin, and that maximum precipitation of antibody occurred when cardiolipin was present with lecithin and cholesterol.

In measuring C fixation, Osler and Knipp showed that rabbit or human syphilitic sera yielded identical reaction curves with three different mammalian cardiolipins, and that dimyristoyl lecithin could substitute for beef heart lecithin in the liposome without reducing its ability to bind IgM and C. They also showed that the lecithins alone had very little anti-complementary activity.

Using dimyristoyl or dipalmitoyl lecithin in the cardiolipin-sensitized liposome system of Osler and Knipp, Humphries and McConnell found that the degree of C fixation increases with the rigidity of the membrane (that is, with cholesterol concentration). A minimum cholesterol content of 35 mol %, however, is required to initiate significant C consumption. It is known from X-ray diffraction and differential scanning calorimetry that phospholipid/cholesterol interaction is maximal at 33 mol %—greater cholesterol concentrations creating cholesterol-cholesterol contacts within the membrane. Humphries and McConnell have used electron spin resonance methods to construct a phase diagram for the lipid systems used which indicates that at greater than 35 mol % cholesterol the cardiolipin becomes strongly immobilised and probably laterally segregated—either in an environment consisting entirely of cholesterol or incorporated in a solid phase of the membrane. The authors suggest that cardiolipin 'receptors' in this state may be able to bind several of the sites on an IgM molecule, thereby distorting the structure of the antibody in the manner required to accommodate the first component of the complement

sequence. This conforms with the hypothesis of C fixation by IgM propounded by Ishizaka and Ishizaka (*J. Immun.*, **102**, 1337; 1969) and supported by electron micrographs of 19S haemolytic antibodies forming loops to bind to nearby sites on a single erythrocyte membrane (Humphrey and Dourmashkin, *Ciba Foundation Symposium on Complement*, 1965).

Returning to the wider implications of their work, Humphries and McConnell, although aware of course that submembranous structures as well as lipid composition are involved in protein mobility in membranes, observe that the conditions of hapten mobility which they find necessary for C-mediated cytotoxic attack may also apply to direct lymphocyte stimulation by these liposomes. As evidence for this they point out that haptens stimulate an immunological response only when they are bound to a protein carrier. Such intriguing parallels will no doubt leave cellular immunologists asking how long it will be before the complementary experiments of manipulating the composition and hence the mobility or function of receptors is accomplished in a living cell. □

Circadian rhythms

by Miranda Robertson

The Dahlem Workshop on the Molecular Basis of Circadian Rhythms was held in West Berlin on November 3-7.

WITH a field in the state that prevails in research on circadian rhythms, the particular format of the Dahlem Konferenzen should have proved an interesting experiment. The Workshops, of which this was the third, are explicitly designed to define the most important problems in a given field and to suggest fruitful directions for further research. Most of the conference time is spent in discussion: instead of being formally presented, background papers are written by and distributed to participants before the beginning of the meeting, and by the end, the members of each of six small groups have been forced into some kind of agreement in the production of a group report on their conclusions.

Agreement is a rare commodity in this field, whose present state has been compared (optimistically?) with that of Mendelian genetics before the discovery of the genetic code in the sense that the "hard" data are phenomenological. It is now very well established that numerous physiological processes, from photosynthesis in plants to motor activity in mammals, have an autonomous more-or-less 24-hour

cycle which can be synchronised with the diurnal light-dark cycle by entrainment. The phenomenology has been enormously extended in experiments on how the various rhythms respond to changes in the phase or the period of the entraining cycle (the Zeitgeber). The task of the workshop participants was to decide on a strategy for discovering what cellular mechanism might give rise to oscillations with the observed characteristics. A group of mathematical modellers tried to decide what those characteristics say about how the clock works in principle and concluded that it is still impossible to distinguish between mechanisms as simple as the "dripping faucet" and as sophisticated as a limit cycle. Indeed, in general the field suffers from a surfeit of models and a dearth of critical experiments. Three categories of non-mathematical model were represented: enzyme models, genetic models and membrane models. The evidence in favour of any of them however tended to be somewhat general: substances which, say, disrupt protein synthesis or alter membrane characteristics sometimes disrupt the circadian rhythm one way or another.

One serious problem facing genetic and membrane modellers is that neither genetic regulation nor membrane function is properly understood in eukaryotic cells.

Another more general problem is that most researchers now agree that it is highly unlikely that there is any single clock mechanism. (Thus the analogy with Mendelian genetics is weak and Colin Pittendrigh's (Stanford) hopes of a "clock phage" doomed to disappointment.) Yet the models are built on the basis of data gleaned from the entire flora and fauna of biological horology, and the confusion is most apparent when anyone attempts to rule out any one possibility. For example, the importance of Ashkenazy's recent discovery is supposed to be that it eliminates genetic mechanisms, since circadian oscillations in enzyme activity seem to persist in red blood cells and

even their isolated membranes. But strictly, it rules out genetic mechanisms only for red blood cells.

This is a curiously tantalising field, in which promising leads seem to turn into blind alleys. The single-celled gonyaulax, for example, manifests circadian rhythmicity in three different physiological mechanisms, all of whose rhythms can be affected by a single mutation. Clock mutants are known in several other species, including two of *Drosophila*. Since both the genetics and the circadian phenomenology of *Drosophila* are so well known, one would expect such mutants to offer a golden opportunity for research.

It does seem, however, that the field would be best served if people stopped looking for "the" circadian clock and focused on that of one particular organism. It may not turn out to be a 'phage' but one well known mechanism could nonetheless prove in the end more illuminating than any number of plausible models. □

Sperm egg interaction

from a Correspondent

The First Symposium of the International Society for Invertebrate Reproduction (ISIR) was held at Calicut on September 10-12, under the auspices of the University of Calicut and the Kerala Academy of Biology. Abstracts have been published in *Invertebrate Reproduction*, (edit by Adiyodi, K. G., and Adiyodi, R. G., Publication Division, ISRPI Secretariat, Calicut University, Kerala, India).

NINETEEN out of twenty animal species on our planet are invertebrates but it is probably no exaggeration that nineteen out of twenty papers published on reproduction deal with vertebrates. As knowledge of reproductive processes and mechanisms is essential for animal management and invertebrates as a group are at least as vital to human welfare as vertebrates, the Calicut Symposium therefore, marks a welcome departure.

Contributions to the symposium covered many aspects of sexuality and reproduction in invertebrates, such as sexual physiology and population dynamics, spermatogenesis, sperm egg interactions, ovarian cycles, chemistry of egg envelopes, nature of accessory sex glands and their secretions, reproductive behaviour and control mechanisms. The ground covered was so diverse that emphasis will be laid here only on contributions in sperm egg interaction.

Richard L. Miller (Temple Univer-



M. CHARBONNIER advocates . . . the theory that people may subsist without food because the nitrogen from the air can be admitted into the circulation system, when the body has been emaciated by long abstinence. Feeding on air is an economical way of keeping soul and body together.

from *Nature*, **13**, 95; Dec. 2, 1875.

sity, Philadelphia) suggested that low-molecular weight species-specific attractants produced by eggs or egg-related structures to draw the sperm toward the egg may occur in most animal groups. Sperm chemotaxis is known to exist definitively only in some sessile Hydrozoa (Cnidaria) and Urochordata. Circumstantial evidence for sperm chemotaxis exists in Calcareia (Porifera), Cephalopoda (Mollusca), Lophophorata (Bryozoa), Chaetognatha, Echinoidea (Echinodermata) and Vertebrata. Chemotaxis of sperm occurs in eggs with simple jelly coat (*Hydractinia*) or with multi-component investments (tunicate eggs). Miller called for more studies on the production characteristics and effectiveness of these chemical messengers and also on sperm response and the effects of these substances on fertilisation rate. R. W. Atherton (University of Wyoming, Laramie) in his contributed paper on neurochemical regulation of sperm motility hypothesised that the effects of neurochemicals on sperm motility in both invertebrates and vertebrates are apparently active through a common pathway linking acetylcholine and acetylcholinesterase to adenylate cyclase activity. Acetylcholine stimulates adenylate cyclase activity and cyclic AMP increases sperm motility.

M. G. Kleve (University of Houston, Texas) described the elaborate centriolar specialisation in spermatids of *Hydractinia* during spermatogenesis and suggested that pericentriolar processes may play an important role in the chemotactic behaviour of the hydrozoan sperm. Wallis H. Clark, Jr., (University of Houston, Texas) said that the cortical rods in the eggs of penaeid shrimps are composed of neutral mucopolysaccharides, and that their release and dissipation are influenced by Mg^{2+} ions, a protease and possibly a phosphatase.

Maturation of starfish oocytes is induced by 1-methyladenine (1-MA) produced by the follicle cells under the influence of a neurohormonal peptide. S. Hirai (Tohoku University, Aomori City, Japan) presented evidence to suggest that cytoplasmic maturation on the oocyte surface responsible for the formation of fertilisation membrane is induced by 1-MA. Mixing of germinal vesicle material with ooplasm, though not a prerequisite for fertilisability of egg cytoplasm is essential for cleavage and in the swelling of sperm nuclei.

Composition of the jelly coat of certain marine eggs has evoked great interest in view of the role of the jelly substance in fertilisation. K. Hotta (Kitasato University, Kanagawa, Japan) discussed the isolation and characterisation of the main sialogly-

coprotein (about 70% sialic acid) from egg jelly coats of three sea urchins, *Pseudocentrotus*, *Anthocidaris* and *Hemicentrotus*. Jelly substance in sea urchin eggs has the ability to agglutinate only homologous sperms, suggesting that species specificity of the jelly substance has a chemical basis. Sialoglycoproteins purified from egg jelly of the three sea urchins by Hotta were distinct and species specific.

Investigations on paedogenesis being few, D. F. Went's (Swiss Federal Institute of Technology, Zurich) report on his success with *in vitro* culture of ovaries of the paedogenetic gall midge, *Heteropeza pygmaea* through successive generations was received with great interest. Sex hormones being practically unknown in insects, it should be possible now to study whether juvenile hormone and ecdysone have any role in determining the sexuality in paedogenetically produced eggs.

To the nearly fifty invertebrate sexual and reproductive biologists it was a week well spent in the ancient city of Calicut, which derives its name from calico it used to export centuries ago. Invertebrate reproductive biology seems to have come to stay as a coherent discipline: the second symposium of ISIR is to be held in the United States in three years time.

Comparative biology of skin

from R. I. C. Spearman

A symposium on the comparative biology of skin was held by the Zoological Society of London on October 30 and 31. The proceedings will be published for the society by Academic Press.

THE skin surface is the main site of contact between animals and a varied and often hostile environment. In addition to having to adapt to external conditions the integument is sometimes involved in skeletal support of the body as in arthropods with their hard cuticles and nematode worms in which the collagenous cuticle has to withstand quite high internal pressure as in a balloon. How species have coped with these varied conditions is reflected in their skin structure and functions which was the underlying theme of a highly successful symposium. This was the first conference to include both invertebrate and vertebrate skins and it provided a useful forum for the exchange of in-

formation between zoologists, entomologists, parasitologists, physiologists and dermatologists. The long periods provided for general discussion at the end of each day were particularly valuable.

K. Simkiss (Reading University) and K. M. Wilbur (Duke University) discussed the functions of mucus secreted by the molluscan skin. Mucus is important in locomotion in land snails and the mucus secreted has been found to have peculiar physical properties; at one stage being watery and at the next stage viscous. The rapid changes in consistency are suggestive of a thixotropic gel, the viscosity of which is effected by agitation of the molecules. It was suggested that the peculiar visco-elastic properties of mucus are important both in locomotion and in its use as a transporting vehicle in association with ciliary activity. Clearly this concept has wider implications extending possibly to mammalian ciliated mucous epithelia. For special purposes such as transport of eggs, chemically different mucus are thought to be secreted and K. S. Richards (Keele University) showed several histochemically different types of mucous cells in the epidermis of oligochaete worms.

Mucus secretion is important in the skins of fish and amphibia but with the appearance of keratinisation this function of skin has been lost in mammals. H. Fox (London University) outlined the epidermal changes in a metamorphosing frog from the ciliated epidermis of the young tadpole to the keratinised epidermis of the adult. As new types of cells are differentiated others are lost by autolysis or by cornification and sloughing of the dead cells.

The sloughing mechanism of the horny layer in toads and its hormonal control was investigated by P. E. Budtz (Copenhagen University). Mineralocorticoid hormone from the adrenal cortex determines sloughing in anura but the ability of the target epidermal cells to respond is dependent on the stage that they have reached in their intrinsic cycle of growth. Thus homographs of toad skin from donors with their moulting cycle out of phase with that of the recipient failed to respond to circulating hormone and moulting of the homograph remained out of phase in subsequent cycles.

E. Johnson (Reading University) spoke of seasonal differences in the hair morphology in deer. The winter coat contains larger medullary air spaces than the summer pelage and is important for thermal insulation in the absence of under fur in cervidae. Possible hormonal control was discussed by J. Ebling (Sheffield University).

At the close of the conference the possibility of forming a society to hold further meetings on comparative skin biology was discussed. □

review article

Colour centres past, present and future

P. D. Townsend*

In this brief review of the role of imperfections in materials, particular emphasis is given to the development of devices involving insulators and semiconductors. The central role of imperfections in modern technology is demonstrated by the fact that the "academic" study of colour centres is essential even in the field of fusion reactors.

SOLID state physics means many things to many people and in various guises forms the backbone of modern technology. Yet the perfect solids on which undergraduate studies of the field are based do not exist and we must manage with imperfect materials. The forms of the imperfections are much more numerous than is suggested by the common division into "point" or "extended" defects. These terms cover a wide range of lattice disorders; point defects refer to errors in the atomic arrangement or impurity atoms which influence a few lattice sites, extended defects to grosser faults in the lattice which include planes of extra atoms, dislocations, precipitates or phase changes. Some typical crystalline defects are listed in Table 1.

The concept of imperfections in a lattice is quite simple but progress in understanding them to the stage at which we can make use of the modified lattice properties to which they lead has been very slow. There are at least two reasons for this. The first is that in practice the problem is often complicated by interactions between different defects. The second is not a practical problem, but arises from the feeling of many academic physicists that imperfection studies are not intellectually challenging. Inevitably this attitude produces undergraduate courses which minimise the importance of defects, and perpetuate the myth of perfect solids. (Even semiconductor physics is often presented as though the impurity atoms were the only defects.) Semiconductors represent just one area of technology which is dominated by imperfections. The list of examples in Table 2 shows that we can separate areas where the major imperfections are the result of impurity atoms; impurities combined with radiation damage; or effects solely of the changes caused by radiation damage to the "pure" lattice. Such a list of applications does not necessarily point the way to future developments; progress so far has often been a mixture of intuition and empiricism. For example, we still do not fully understand the mechanism of the photographic process although it has been in use for over a century¹.

To emphasise the property changes which can result from imperfections, and the devices which exploit them, I will select examples from colour centre research²⁻⁶. This field is commonly regarded as a fringe area of solid state physics concerned with the effects of X rays on alkali halides. In fact the issues range more widely. For example, among the suggested methods for obtaining energy from

nuclear fusion is a system which uses optics and pulsed lasers to trigger the thermonuclear reaction. This entire system will be in a radiation environment and, as I shall discuss, will rapidly fail unless the problem of colour centre formation is overcome.

Nature of colour centres

Colour centres represent a special case of defects found in transparent materials. Perfect crystalline solids are commonly described in terms of bands of allowed energy states which extend throughout the crystal. Insulators, semiconductors and metals differ according to this picture only, in the relative concentrations of electron and unfilled states which exist in the highest occupied band of levels. Imperfections, either intrinsic (that is, from disorder of the lattice atoms or the occupancy of lattice sites) or extrinsic, from the presence of impurity atoms, introduce localised energy levels and if these lie within the forbidden energy gap they provide a means of controlling the electrical properties of the solid. For example, the highest occupied electron level of an impurity atom may occur between the valence and conduction bands. Several such examples of interband energy levels are shown in Fig. 1. In the first example, *A*, the electron can be promoted to a higher localised state of the defect but in example *B*, the localised electron can enter the conduction band. This is of great importance for semiconducting materials such as silicon where the conduction band is essentially empty at room temperature but can be controlled by electrons which are optically or thermally stimulated from defects of type *B*. Figure 1 also indicates electron transfer to a deeper level which could be accompanied by luminescence.

Inter-band energy levels therefore influence many solid state properties such as conductivity, luminescence, chemical reactions, corrosion or optical absorption. The presence of the defects will be equally apparent in mechanical properties or in phase transitions.

Insulators have large energy gaps so are transparent to visible radiation. Optical transitions of either type *A* or *B* of Fig. 1 will produce optical absorption at the resonance energy, however, in a solid this is not a line absorption process as there may be coupling to the phonon spectrum. Consequently the absorption lines are broadened and the crystal may visibly change colour, hence the term colour centres to describe defect levels in insulators. Natural examples are ruby and sapphire which are the same host lattice (Al_2O_3) with impurity ions of either chromium or titanium. The same principle underlines the performance

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Table 1 Some typical defects in solids

Vacancies	Atoms missing from lattice sites. A variety of charge states may exist in insulating materials.
Interstitials	Atoms situated in between normal lattice sites
Impurities	All possible types and configurations.
Clusters, precipitates	Either planar or three dimensional. in the same or new crystal phases.
Dislocations	Faults in the order of crystal planes.
Voids	Large aggregates of vacancies.
Bubbles	Vacancy clusters with inclusion of gas, for example from α particles.

of stained glass or colour filters which make use of optical absorptions bands ranging from the infrared to the ultraviolet. We should appreciate that the term "colour centre physics" is artificial as electronic transitions such as *B* or *C* of Fig. 1 may be described as photoconductive or luminescence phenomena.

The techniques for sensing the defects are often very sensitive and where it is possible to use electron spin resonance (ESR) or electron nuclear double resonance (ENDOR) the interactions with crystalline field and nuclear spins provide detailed information on the imperfection. Even if the defect does not contain an unpaired spin it is possible to follow changes in charge state and investigate thermal instability and defect interactions by the dispersive method of optical absorption or luminescence spectroscopy. With techniques available for detecting defects in parts per 10^{10} of normal atomic sites, all materials reveal departures from chemical purity. The impurity level in most electronic devices is, however, irrelevant if the impurity ions do not take part in the process for which the device is designed. Therefore the approach is often to purify as far as possible, and then add a high concentration of known impurities to overshadow the residual effects of trace impurities. This can achieve a limited practical objective but may not, of course, increase our understanding of the role of defects, nor allow us to proceed to the manufacture of a similar device.

Classical colour centre studies have concentrated on intrinsic defects in the alkali halides. The reasons for this are partly historical. It was possible to obtain single crystals with a simple structure, a wide band gap and visual effects of dissociation from exposure to X rays. Furthermore, the size of the defects is a function of the lattice parameter so that the transition energies scale with the sequence of alkali halides⁷. The most famous example, the F centre (an electron trapped at a vacant halogen lattice site), follows this pattern, and was positively identified by ESR and ENDOR. It is also a classic example of an electron in a

box and by solving the simplest of Schrödinger equations we can predict the position of the optical absorption band. At this point it would be nice to say that the F centre is typical of the other intrinsic defects in alkali halides. In reality we have only established defect models for half a dozen centres in spite of forty years of worldwide research, and with hindsight much of the literature is valueless because of problems with impurities. Nor does this apply only to work with alkali halides or the results published in the distant past. Rather, it is exceptional to study a colour centre problem which is not qualified by statements about unknown imperfections. Alkali halide research happens to have reached a stage at which we can sense the weaknesses in the data but few other materials are so well understood. The only universal cure is to raise the art of crystal growing to a status at which it will attract the most able and dedicated scientists. All too often this phase of a research programme is left to someone else because it is quicker to produce "new" results from available material. (Maybe I should add that I too am guilty of this attitude.) On the occasions on which I have consulted crystal growing manufacturers they have been helpful but for financial reasons do not wish to develop material for an uncritical market.

Imperfections and practical devices

We shall now look in more detail at three classes of device which exploit imperfections. The commonest depend on impurities. Intrinsic defects or modifications by radiation damage have only received serious attention since the advent of nuclear reactors. Frequently the impurities are added to charge-compensate other imperfections, as with semiconductors. For an optical example one may cite the case of fused silica which has useful properties in both the infrared and the ultraviolet. Here the presence of OH radicals introduces an infrared absorption band but produces the benefit that the OH also removes the absorption in the near ultraviolet region. Manufacturers of paints apply the same principle to produce whiter paints. A standard problem is a paint which contains titanium dioxide, which forms a range of compounds from TiO_2 to Ti. A deficiency of oxygen produces a yellow product as the band gap is effectively narrowed. Charge compensation by ions of lower valency for example Al restores the transparency to the material by sharpening the optical absorption edge and removing the discoloration.

To digress a moment; we tend to expect compounds to obey Dalton's laws of gas composition and are surprised by materials like TiO_x ($0 < x \leq 2$). Dalton's laws need not apply in solids and it is quite possible to construct a lattice with an ordered array of vacant lattice sites. It is only our pre-

Table 2 Devices and fields of study which are dominated by the role of imperfections

Impurity related	Impurity plus radiation damage	Radiation damage
Semiconductors	Corrosion	Academic studies of colour centres
Phosphorescence	Sensitivity of explosives	Dark trace oscilloscopes
Luminescence	Photographic process	Information storage
Photoemission	Photochromic glass	Photolysis
Photoconductivity	Photolithography	
Solid state lasers	Radiation dosimeters	
Coloured gem stones	Ion implanted semiconductors	
Optical filters	Ion implanted optical waveguides	
Optical windows		
Stained glass		
Paints		

Imperfections may be introduced as chemical impurities, by displacement of atoms from their normal lattice sites (radiation damage), or by a combination of the damage and impurity effects. The three columns provide examples of each.

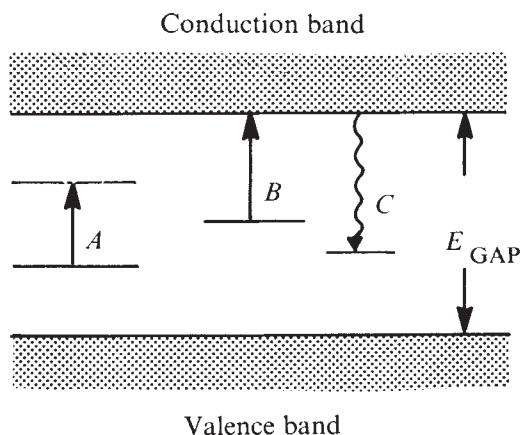


Fig. 1 The energy level diagram of an insulator which has localised imperfections. Transitions A or B correspond to optical absorption, C is a radiative transition.

judice that describes these as imperfections. For this we suffer, as we then express the composition in rational numbers (for example $\text{W}_{50}\text{O}_{148}$) and are further surprised when such compounds have a simple unit cell.

Charge compensation can be reversed, for example by light which ionises one defect and transfers the charge to a second site. Consequently the colours of many paints and dyes are unstable in sunlight. Such materials are termed photochromic⁸. The positive advantages of the light sensitivity are in photochromic glasses which darken in sunlight, or in information storage systems. Figure 2 outlines a suitable scheme of energy levels for an erasable information store. To write the data an energy W excites (writes) an electron from level (i) and transfers it to a different defect. The population of the ground state of (ii) is read by an energy R and erasure is achieved with E . There are many variations to this scheme. One example is SrTiO_3 doped with Mo^{6+} and Fe^{3+} on Titanium sites⁹. The charge transfer involves conversion of $\text{Fe}^{3+} \rightarrow \text{Fe}^{4+}$ and $\text{Mo}^{6+} \rightarrow \text{Mo}^{5+}$.

Thermal erasure is an alternative which is used in photochromic windows and "automatic" sunglasses. Again there is a wide choice of glass and impurity but at least one commercial material is based on small islands (<10 nm diameter) of AgCl in the glass matrix. The rationale for this is clear: AgCl has an optical absorption band in the near ultraviolet which initiates the photographic process. It is less obvious that it also has the advantage of being reversible. For this is not merely a charge transfer but decomposition of the lattice. The Ag^+ ions convert to Ag^0 ions which are no longer bonded to migrate through the lattice to form the latent image as specks of colloidal silver for example Ag^0 . In the photochromic glass the decay product of the AgCl cannot leave the island of AgCl and enter the glass matrix which is in contrast with the reaction of Cl from AgCl with the gelatin of photographic emulsions. The close presence of free Ag and Cl in the glass example means the process is reversible.

The current view of the photographic process is slightly more complex than this, however, and requires the addition of impurity atoms and a change in chemical stability of grains with latent images within them. In broader terms the photographic process embodies all the principal kinds of imperfection in solids. To summarise they are: (1) An initial charge transfer and chemical stability controlled by impurities. (2) Intrinsic defects produced by neutron, proton or other kinds of radiation and which react with themselves and existing imperfections. (3) The larger defects which cluster or precipitate out of the lattice tend to be more stable than point defects. These all lead to a modified

crystal with an overall chemical stability which differs from the original sample.

An understanding of these problems opens new avenues of research for areas as apparently diverse as photolysis, corrosion, solubility, or catalysis as well as the formal solid state studies. Imperfections may increase or reduce chemical stability and photolysis. Among the more spectacular examples it has been found that several compounds undergo photolysis, explosively, when the impurity is reduced. Conversely radiation damage can enhance solubility. Here examples range from the conversion of an opal glass to a lithium metasilicate by photons, the use of photon and electron beams in lithography for miniature surface devices and changes in chemical solubility brought about by ion implantation. In all three cases, the solubility can be changed so much that the patterned area can be etched with negligible attack to the remaining areas. Each case however provides us with a different conceptual approach. The metasilicate case involves a chemical phase change; in the lithographic example, the electrons produce ionisation and local rebonding which may also imply isolated point defects, whereas the ion implantation provides disordered regions along the ion tracks and very high concentrations of displaced atoms. The injection of ions at high velocity may also prove useful in changing the chemical imperfection properties of the solid as is evidenced by its widespread use in the manufacture of integrated electronic circuits. A related field of optical circuitry of surface waveguides, acoustic wave devices, modulators and couplers is now developing and also links together the problems, and advantages, of colour centres and ion implantation¹⁰⁻¹². These examples seem to have brought us up to the present state of the art and I shall conclude with a tentative look at a future problem.

Colour centres and fusion reactors

Fission reactors produce a range of fission fragments with a wide spectrum of energy and mass which cause damage in the structural materials. In general terms the radiation damage is to be expected and to some extent is allowed for in the reactor design, either by the choice of materials which are relatively unchanged or, in extreme cases, by allowing for replacement sections. With each new reactor, however, new problems arise because of the higher operating temperatures, higher fluxes or reactivity of the coolants.

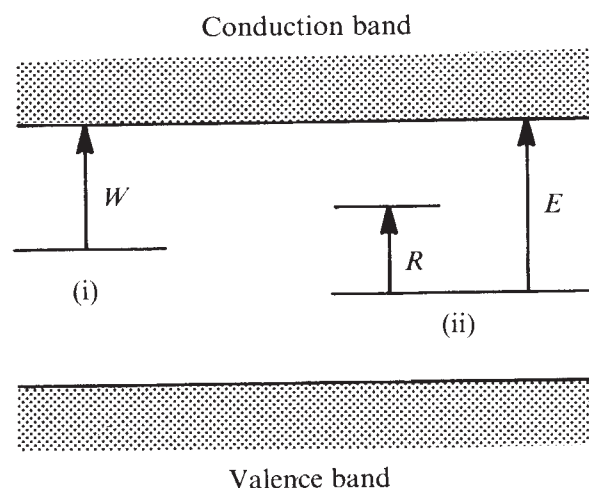


Fig. 2 An energy level scheme which would be suitable for information storage. Writing is performed by transfer of an electron from defect (i) to defect (ii). Read measurements are made by sensing the population of the defect (ii) ground state by the optical transition R. Erasure proceeds by transfer to defect (i) by energy E. In this example all three optical processes are in distinct energy regions.

Many of these problems will also exist in fusion reactors and an obvious case to consider is what would happen if the fusion events are triggered by pulses of laser light. Laser beams deposit energy in a glass by absorption or scattering. If the pulses of light are sufficiently intense then the rate of energy deposition will cause the glass to explode¹³. For this reason one is very careful to remove strain or refractive index gradients from the glass and in normal environments the main concern is with the polish of the surface layer. Mechanical polishing leaves residual strains which may set an upper limit to the power density before fragments of material explode from the surface. Chemical or ion beam etching of this surface strain can raise the threshold level from 2 to 10 GW cm⁻².

In a radioactive environment fission tracks, colour centres or large scale aggregates of defects can all produce the same catastrophic events in the bulk of the specimen. Further, because the damage is unlikely to be uniform through the material there is also mechanical strain. In early fission reactors the solution was to heat the materials and anneal the damage. In later reactors this has not always been possible because of the risk of enhancing the formation of large stable defects such as voids or gas bubbles. The research on voids has also indicated that they are produced at a higher rate at high fluxes (this is the situation in a pulsed fusion system). In short, we shall need glass windows, and possibly laser materials, which are in close proximity to the fusion vessel which must not (1) distort (2) colour (3) accrete large scale defects or (4) increase the surface

scatter. Problems (1), (2) and 3 are fairly intractable, (4) is certainly insoluble because sputtering of surface atoms is a consequence of radiation damage. In an insulating material the problem can be further aggravated by charge build up below the surface followed by explosions in which blocks of material are ejected. This would cause laser scatter and destruction of the glass by the subsequent laser pulses.

Clearly with a little thought we could find other potential problems. Instead, I would suggest these problems are not insurmountable. For example the oxide glasses with a large inherent vacancy concentration (for example TiO₂) are essentially self-annealing and remarkably stable to radiation damage. Similarly, if this type of material could be made of high enough electrical conductivity, yet remain transparent, then the problems of surface charge explosions might also be avoided.

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articles

Linkage disequilibrium between *H-2* and *t* complexes in chromosome 17 of the mouse

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Mice with t factors belonging to the same complementation group carry similar, if not identical, H-2 haplotypes although these factors were derived from widely separated geographical areas. This association between t and H-2 complexes suggests more than a casual relationship between the two complexes, at least at the population level.

CHROMOSOME 17 of the mouse carries two of the most puzzling systems in mammalian genetics, *H-2* and *t* (refs 1-3). The question whether the presence of two such highly sophisticated systems on the same chromosome is fortuitous or whether the systems could be genetically interrelated has been raised

repeatedly²⁻⁶. In this communication we demonstrate at least one level of interrelation between *t* and *H-2* by showing the presence of a strong linkage disequilibrium in the mouse population between the two complexes.

While studying the polymorphism of serologically detectable *H-2* antigens among wild mice⁷ we *H-2* typed strains carrying various *t* factors because most of these factors have been extracted from wild mouse populations, and there was a good chance that they carried the wild-derived *H-2* haplotypes. The *t* bearing strains were from our mouse colony or that of Dr Dorothea Bennett at Cornell University Medical College. The former were strains at various stages of transfer of individual *t* factors (originally obtained from the late Professor L. C. Dunn) on the inbred background of strain C57BL/10Sn (B10); the

Table 1 H-2 antigens in *t*-bearing strains of mice.

Factor	H-2 haplotype	1	2	3	4	5	6	7	8	11	13	23	25	31	105	106	107	108	109	Ia antigens 101
<i>t</i> ¹²	<i>t</i> ¹²	—	—	—	—	5	—	—	—	—	—	—	—	—	—	106	107	—	—	—
<i>t</i> ^{w32}	<i>t</i> ¹²	—	—	—	—	5	—	—	—	—	—	—	—	—	—	106	107	—	—	—
<i>t</i> ^{w2}	<i>w</i> ⁵	—	—	"3"	—	—	"6"	C	8	—	—	—	—	31	105	—	107	—	—	—
<i>t</i> ^{w8}	<i>w</i> ⁵	—	—	"3"	—	—	?	.	.	—	.	—	—	31	105	—	107	—	—	—
<i>t</i> ⁰	<i>w</i> ⁵	—	—	"3"	—	—	?	.	.	—	.	—	—	31	105	—	107	—	—	—
<i>t</i> ¹	<i>w</i> ⁵	—	—	"3"	—	—	?	.	.	—	.	—	—	31	105	—	107	—	—	—
<i>t</i> ⁶	<i>tw</i> ¹	—	—	—	—	C	—	—	—	—	"13"	—	—	—	—	—	—	108	—	—
<i>t</i> ^{w1}	<i>tw</i> ¹	—	—	—	—	C	—	—	—	—	"13"	—	—	—	—	—	—	108	—	—
<i>t</i> ^{w71}	<i>tw</i> ¹	—	—	—	—	—	—	.	.	—	.	—	—	—	—	—	—	108	—	—
<i>t</i> ^{w73}	<i>tw</i> ¹	—	—	—	—	?	—	.	.	?	.	—	—	—	—	—	—	108	—	—
<i>t</i> ^{w12}	<i>tw</i> ¹	—	—	—	—	?	—	.	.	?	.	—	—	—	—	—	—	108	—	—
<i>t</i> ^{w5}	<i>tw</i> ⁵	"1"	—	—	—	5	—	—	—	11	—	C	25	—	—	—	—	—	109	101
<i>t</i> ^{w75}	<i>tw</i> ⁵	—	—	—	—	?	—	.	.	11	.	—	25	—	—	—	—	—	109	—

(Antigens H-2.2, 4, 9, 15, 16, 17, 18, 19, 28, 30, 32, and 33 were found to be absent in all tested *t* strains.)

A dot indicates not done. Quote marks indicate a similar but not identical antigen. C, cross-reacting antigen. A question mark indicates that presence or absence of an antigen is uncertain.

latter were mostly outbred animals maintained as balanced lethal lines in combination with the *brachyury* chromosome (*T/t*). The detailed results of this typing will be presented elsewhere; here we summarise the data and demonstrate a correlation between *t* complementation groups and H-2 haplotypes.

The first step in H-2 typing was to use cells from *t* strains as targets for haemagglutinins and/or cytotoxins produced against known H-2 antigens. This provided limited information about the *t* strains, since few positive reactions were observed. Most known H-2 antigens were absent in the *t* strains; however, those present had a distribution more or less coinciding with the classification of *t* factors into complementation groups (antigens 1–31 in Table 1). Thus, *t*¹² and *t*^{w32}, two members of the *t*¹² group, both carry H-2.5 and *t*^{w5} and *t*^{w75}, two members of the *t*^{w5} group, carry antigens H-2.11 and H-2.25. In addition, *t*^{w1}, *t*^{w71} and *t*^{w12}, all members of the *t*^{w1} group and *t*^{w73}, which seems to define a new complementation group¹³ also lack most known H-2 antigens. The two semi-lethal factors, *t*^{w2} and *t*^{w8} have a similar antigenic pattern. But the factors belonging to the *t*⁰ group, *t*⁰, *t*¹ and *t*⁶, possess different antigenic patterns.

Because most known H-2 antigens were absent in *t* strains, we tested whether these strains carry new, so far unidentified, H-2 antigens. We immunised inbred strains and their hybrids with tissues from *t/t* heterozygotes sharing an H-2 haplotype (the one present in the + chromosome) with the recipient. We produced five antisera (Table 2) which were particularly useful in characterisation of *t* strains. Analysis of these antisera led to a definition of five new antigens, shown by linkage tests to be controlled by genes closely linked to H-2 (data not shown). Antigens 101 and 106 to 109 were absent in all inbred strains, and H-2.106, 107 and 108 apparently occurred with a relatively low frequency among wild mice²⁴. As Table 1 shows, however, the antigens are shared by some of the *t* strains, and this sharing, to a certain degree, parallels the classification of *t* factors into complementation groups.

One possible explanation of this peculiar antigenic similarity among members of the same complementation group is that our antisera detect not H-2 antigens, but products of the *I* loci. To test the relationship between the *t* factors and the new antigens, we first established the tissue distribution of the antigens. As Table 3 shows, the distribution of H-2.106, H-2.107 and H-2.108 is that of classical H-2 antigens since reactivity against antigens was cleared or reduced by spleen, lymph nodes, thymus, liver, kidney, heart, brain and erythrocytes. In contrast the antigen detected by the cytotoxic reactivity of H-27 is present only on spleen and lymph node cells, and the antiserum reacts only with bone-marrow-derived (B) lymphocytes and not thymus-derived (T) lymphocytes (Fig. 1). The tissue distribution of this antigen is thus indicative of an *I*a antigen⁸, and this antigen has been designated Ia.101. H-27 also has reactivity in the PVP-haemagglutination test in which it detects H-2.109. As this reactivity is weak, however, the assignment of H-2.109 should be considered tentative.

We also tested the molecular relationship of the new antigens to the few known H-2 antigens demonstrable in *t* strains. This test was based on the technique monitoring redistribution in the cell membrane of antigens combined with two layers of antibodies⁹. A typical experiment of this sort is shown in Table 4. In this experiment, pretreatment of +H-2/*t*¹²H-2/*t*¹² spleen cells with K-46 (anti-H-2.5) induced resistance to H-12 (anti-H-2.106) but not to the anti-H-2^a sera, K-304 (anti-H-2.4) and K-25 (anti-H-2.31) and vice versa. It thus seems that H-2.5 and H-2.106 are on the same molecule, as they are redistributed in the cell membrane together.

On the basis of these data, we conclude that antigens H-2.106 to H-2.109 are not the products of the *t* loci but typical H-2 antigens; 101 seems to be an *I*a antigen. The presence of the new antigens on adult somatic tissues makes it unlikely that they are products of *t* loci, which are expressed only during embryogenesis and on spermatozoa¹⁰.

If one accepts this conclusion, members of the same complementation group of *t* factors must have either identical or very

Table 2 Antisera detecting H-2.106, H-2.107, H-2.108, H-2.109 and Ia 101

Code No.	Recipient	Donor	H-2 haplotype combination†	Antigens detected
H-12	(B10 × A)F ₁	(B10 × T/ <i>t</i> ¹²)NT‡	(<i>b/a</i>) <i>b/t</i> ¹²	H-2.106, H-2.107
H-25	(B10.D2 × D2.GD)F ₁	<i>t</i> ^{w2} / <i>t</i> ^{w2}	(<i>d/g2</i>) <i>tw5/tw5</i>	H-2.107
H-11	(B10 × A)F ₁	(B10 × T/ <i>t</i> ^{w1})NT	(<i>b/a</i>) <i>b/tw1</i>	H-2.108
H-26	(B10 × A)F ₁	(B10 × T/ <i>t</i> ⁶)NT	(<i>b/a</i>) <i>b/tw1</i>	H-2.108
H-27	(B10 × C3H)F ₁	(B10 × T/ <i>t</i> ^{w5})NT	(<i>b/k</i>) <i>b/tw5</i>	H-2.109, Ia.101

† Recipient in parentheses.

‡ Normal tail.

similar alleles at the loci coding for serologically detectable *H-2* antigens. Our preliminary data on mixed lymphocyte reaction typing of *t* strains parallel the serological data and thus suggest that the intra-group similarity encompasses a large portion of the *H-2* complex. The significance of this finding becomes apparent when one takes into account the origin of the individual *t* factors. For example, t^{w1} was extracted before 1955, from a wild mouse captured in the New York area¹¹; t^{w71} was obtained from a wild mouse trapped in 1968 in Denmark¹²; and t^{w12} was derived from a wild mouse captured in California before 1956 (ref. 13). All three factors belong to the

complementation groups) and that these chromosomes have been perpetuated in mouse populations for a very long time.

This interpretation of *t* population behaviour suggests a far greater role for interdemographic migration than was originally postulated. Demes have been believed to be virtually impenetrable to migrants and the migration rates were thought to be extremely low¹⁵. If the only source of *t* factors is, however, immigration, then the migration rates must be quite significant.

Our data also have an important implication concerning the relationship between the *t* and *H-2* systems. The data indicate that, at the population level, the two systems are interrelated in

Table 3 Tissue distribution of H-2.106, H-2.107, H-2.108, and Ia.101

Antigen	Reciprocal of titre after absorption with the following tissues*															
	Spleen t strain†	B10	Lymph nodes t strain	B10	Thymus t strain	B10	Liver t strain	B10	Kidney t strain	B10	Heart t strain	B10	Brain t strain	B10	Erythrocytes t strain	B10
H-2.106‡	—	128	—	128	—	128	—	128	—	128	—	128	—	128	—	128
H-2.107§	—	960	—	960	—	960	—	1,920	—	1,920	120	960	240	960	—	960
H-2.108 ¶	—	512	—	512	128	512	—	512	128	512	266	512	256	512	256	512
Ia.101	—	8	—	16	8	8	16	16	8	16	16	16	16	16	8	16

* Absorption was done with one part packed tissue: two parts diluted antiserum for 30 min at room temperature, then 30 min at 37 °C.

† Tissue used for absorption was from the strain against which the absorbed serum was then tested.

‡ H-12 was diluted 1:64, absorbed, then tested against $+/t^{12}$ lymphocytes in a cytotoxic test⁸ (an unpublished work with K. Artzt and D. Bennett); titre was the lowest dilution giving 50% cell lysis.

§ H-25 was absorbed first with B10.BR, then diluted 1:10 and absorbed with the above tissues, and tested against t^{w2}/t^{w2} erythrocytes in a PVP-haemagglutination test²⁴; titre was the lowest dilution giving an agglutination score of 1.

¶ H-11 was diluted 1:64, absorbed, then tested against $+/t^{w1}$ lymphocytes in a cytotoxic test; titre was the lowest dilution giving 50% cell lysis.

|| H-27 was diluted 1:2, absorbed, then tested against $+/t^{w5}$ lymphocytes in a cytotoxic test; titre was the lowest dilution giving 50% cell lysis.

same complementation group and all carry *H-2* haplotypes characterised by the presence of antigen H-2.108. Thus, individual members of the same complementation group are unrelated in terms of place and time of their extraction from wild mouse population, but they have very similar *H-2* haplotypes. Similar relationships exist among members of other complementation groups. The only serious exception is the t^0 group: the t^6 factor which is classified as a member of the t^0 group, has an *H-2* haplotype which resembles that of the t^{w1} group, while t^0 and t^1 have haplotypes similar to *H-2^{w5}*. But, t^0 , t^1 , and t^6 are among the factors maintained for the longest time in the laboratory, and therefore they might have been modified by recombination. Supporting the contention is the fact that, for example, t^6 has a relatively low transmission ratio (0.67 C. H., unpublished). (Factors extracted from wild mice usually have transmission ratios of > 0.9 ; factors derived by recombination from t^{wild} factors usually have much smaller ratios.) It is possible that t^6 is a recombinational derivative of another factor (t^{w1}), while $t^0(t^1)$ or a *t* factor from which t^0 is derived may be responsible for the dispersal of the *H-2^{w5}* haplotype among wild mice.

The finding that *t* chromosomes within the same complementation group carry similar *H-2* haplotypes has important implications with respect to the origin of *t* factors and their role in maintaining *H-2* polymorphism. There is a wealth of experimental data indicating that wild mice live in small, relatively isolated units or demes with a social structure dominated by a single male (review in ref. 1). Because of the lethality of *t* factors, these factors can never become fixed in any particular deme and the *t* factors already present in a deme are eliminated by random genetic drift, and new factors are reintroduced into the demes¹⁴. The question then arises: what is the source of the reinfected *t* factors? Do the factors represent new mutations (in the broadest sense of the word) or are the same factors reintroduced over and over by interdemographic migration? Our data strongly suggest that the latter of the two explanations is correct. It seems that there is only a limited number of different *t* chromosomes among wild mice (perhaps fewer than there are

that certain *t* factors are associated with specific *H-2* haplotypes. In other words, the *t* factors and *H-2* haplotypes show a strong linkage disequilibrium. What could be the significance of this disequilibrium? The simplest interpretation is that the disequilibrium is the consequence of the *de facto* close linkage of the two complexes (W. Bodmer, personal communication). As discussed by Thomson *et al*¹⁶, linkage disequilibrium between two closely linked loci can persist over long periods if a relatively small selective effect is acting on one of these loci. For example, in a typical Northern European human population, the frequency of the *HLA1* allele is 0.16 and of the *HLA8* is 0.13. The frequency of the *HLA1*, 8 haplotype is 0.08, which corresponds to linkage disequilibrium $D = 0.06$. At the same time, the distribution of this haplotype throughout Europe suggests that it has been present at reasonable frequencies since pre-agricultural times. The association between certain *t* factors and specific *H-2* haplotypes could be an analogous example of a persistent linkage disequilibrium.

This interpretation, however, does not explain the nature of the selective effect maintaining the linkage disequilibrium. In contrast to disequilibrium in the *HLA* system, where selection acts on loci that are physically close to each other on the chromosome and where the haplotypes that are in disequilibrium show no evidence of crossing over suppression, an active suppression is a vital part of the mechanism maintaining the *t-H-2* disequilibria. Furthermore, to achieve this suppression, the mouse apparently pays a high price in the form of homozygous lethality of the embryos. One would therefore expect that there is a good reason for this costly invention. What might this reason be? We propose that the selective advantage of the linkage disequilibrium lies in the production of genetically superior sperm. It is known that the loci of the *t* complex are expressed on sperm and that they affect sperm behaviour¹⁷. The sperm carrying a *t* mutation has a much higher probability of fertilising an egg than the normal or the *T* sperm and the consequence of this effect is the distorted segregation observed in a $t/+ \times +/+$ mating. Circumstantial evidence indicates that a group of loci between *T* and *H-2* in chromosome 17 influences

the physiological properties of the sperm^{18,19}. One can therefore envision that certain combinations of alleles at these loci result in a physiologically superior sperm, and that, for this reason, these combinations are selected for in natural populations of the mouse. When a structural aberration suppressing crossing over occurs in the chromosome carrying an advantageous combination of alleles, the alleles are permanently locked together and the chromosomal segment transmitted intact from generation to generation. Theoretically, such an advantageous combination should spread rapidly throughout the population and replace all less advantageous combinations of alleles. In reality, however, this does not happen because the chromosomal aberration also has a deleterious effect on its bearer in that it causes homozygous lethality of the embryos and the lethal effect then balances out the advantageous sperm effect. This interplay of the two opposing forces is possible because of the subdivision of wild populations into demes in which genetic drift¹⁵ and inbreeding²⁰ keep the frequency of the *t* factors below deterministic expectations²¹. The result of the interplay is that the interlocked combination of alleles (a particular *t* factor) is maintained in the population in spite of the deleterious effect, but because of this effect it is prevented from completely taking over the population. Consequently, a particular *t* factor persists in the population with a stable frequency determined by the interaction between the two opposing forces, segregation distortion, and lethality. In cases where the crossing over suppression effect is strong enough to encompass the *H-2* complex, a particular *H-2* haplotype is locked together with a particular *t* factor, and a linkage disequilibrium of this combination then develops. In the history of the mouse population the locking together of the alleles occurred independently several times and involved different allelic combinations of parts of chromosome 17 and different *H-2* haplotypes.

According to this concept most complementation groups are the result of independent occurrences of the locking together event. On the other hand, the various *t* factors of the same complementation group are all derived from the same locking together event, although they might have subsequently differentiated by mutation. Some members of a complementation

Table 4 Susceptibility of $+H-2^d/t^{12}H-2^{12}$ spleen cells to cytotoxic killing after anti-*H-2* and GAMIG treatment

Pretreated with*	Tested with	Dead cells at dilution of antiserum (%)		
		1:2	1:4	1:8
GAMIG (1:4)	H-12	88	85	80
H-12 + GAMIG (1:4)	H-12	25	25	25
K-46† + GAMIG (1:4)	H-12	38	30	30
K-304‡ + GAMIG (1:4)	H-12	85	82	80
K-25§ + GAMIG (1:4)	H-12	88	80	72
GAMIG (1:4)	K-46	70	60	50
H-12 + GAMIG (1:4)	K-46	30	35	35
K-46 + GAMIG (1:4)	K-46	25	25	20
K-304 + GAMIG (1:4)	K-46	70	65	60
K-25 + GAMIG (1:4)	K-46	65	60	50
GAMIG (1:4)	K-304	90	90	90
H-12 + GAMIG (1:4)	K-304	88	85	80
K-46 + GAMIG (1:4)	K-304	85	85	80
K-304 + GAMIG (1:4)	K-304	25	25	25
K-25 + GAMIG (1:4)	K-304	85	85	80
GAMIG (1:4)	K-25	85	85	80
H-12 + GAMIG (1:4)	K-25	85	80	80
K-46 + GAMIG (1:4)	K-25	85	80	80
K-304 + GAMIG (1:4)	K-25	85	85	80
K-25 + GAMIG (1:4)	K-25	25	25	20

The cytotoxicity test was performed as described by Hauptfeld *et al.* (ref.8). GAMIG, goat anti-mouse Ig (γ , κ), was produced and supplied by Dr E. S. Vitetta, University of Texas. Haplotype *H-2*^d possesses *H-2.4* and *H-2.31*.

* All anti-*H-2* sera were used undiluted. Cells pretreated with GAMIG and tested with normal mouse serum and complement had a background of 15% cell lysis.

† K-46: (HTG $\times$ B10.D2)F₁ anti-B10; anti-H-2.5.

‡ K-304: (A.BY $\times$ B10.AKM)F₁ anti-B10.A; anti-H-2.4.

§ K-25: (A $\times$ B10.A)F₁ anti-B10.D2; anti-H-2.31.

group, however, might have been derived from a *t* factor of a different complementation group by a rare recombination within the *t* complex.

Whether the *H-2* complex has any evolutionary homology to the *t* complex is not known. But, the finding that antigens coded for by one of the *t* loci have a similar molecular weight as the classical *H-2* antigens²² suggests that such homology might indeed exist. In any event, it is clear that the *H-2* complex, at least at the population level, is included in the sphere of action of *t* and thus is a part of the *t* supersystem.

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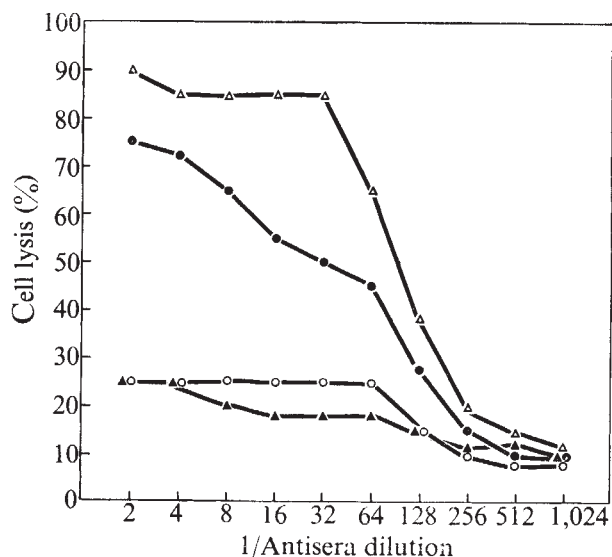


Fig. 1 Cytotoxic reactivity of anti-Ia.101 and anti-Thy-1.2 (θ -C3H) sera with thymus-derived lymphocytes (T cells) and bone-marrow-derived lymphocytes (B cells) from *t*^{ws} spleen. T cells were obtained by filtration of spleen cells through nylon fibre columns²³. B cells were prepared by the method of Hauptfeld *et al.*⁸. The cytotoxicity test was performed as described by Hammerberg *et al.*⁸ (unpublished). ○, anti-Thy-1.2 reactivity against B cells from *t*^{ws} spleen; ●, anti-Ia.101 reactivity against B cells from *t*^{ws} spleen; △, anti-Thy-1.2 reactivity against T cells from *t*^{ws} spleen; ▲, anti-Ia.101 reactivity against T cells from *t*^{ws} spleen.

Evidence for cell surface control of macronuclear DNA synthesis in *Stentor*

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In cell grafts, Stentor macronuclei associated with separate regions of cell surface can be made asynchronous with regard to morphology and DNA synthesis even though they demonstrably share a common endoplasm. These results suggest a mechanism for nuclear differentiation within a single cytoplasmic compartment, based on cell surface differences.

THE baffling phenomenon of nuclear differentiation within a single cytoplasmic compartment occurs in many animal and plant cell types (for example, developing eggs¹, grasshopper neuroblast cells², pollen grains³, cells of the developing stomatal complex³, ciliates⁴ and foraminiferans⁵). In most ciliates, nuclear differentiation occurs during the formation of micro- and macronuclei from a single zygote nucleus during sexual reproduction (conjugation). The two types of nucleus, which differ strikingly in morphology and nucleic acid content, develop in different regions of the cell and centrifugation experiments have shown that in *Tetrahymena*⁶ and *Paramecium*⁷ the developmental fate of the presumptive nuclei can be altered by displacing them.

narrow striping on the ventral cortex. The stages of oral development have been assigned numbers 1–8 (ref. 10), with each stage lasting about 1 h at 20 °C. While the primordium is developing, the chain macronucleus first coalesces (stage 5) into a compact mass (stage 6), then elongates (stage 7) and renodulates so as to double the number of nuclear nodes in preparation for division. After a G₁ period of 2–12 h, macronuclear DNA synthesis is continuous throughout interphase (36–48 h) and stops at or near the time of nuclear coalescence¹¹. Similar morphological and synthetic events of macronuclear replication also occur if oral development is induced during interphase by removing the feeding structures; they are more conveniently studied during this process (regeneration, Fig. 1) because of the ease with which large numbers of cells can be obtained in any given stage. My experiments therefore involved regenerating stentors.

A stentor in early regeneration continues to develop when grafted to a morphostatic cell of comparable size; when it reaches stage 6 and undergoes nuclear coalescence it induces this event in its graft partner as well¹². Nuclear coalescence is, however, usually not induced if a thin, transverse disk taken from a third (morphostatic) stentor is interposed as a barrier between the two main graft components. This operation (Fig. 2) produces visible double rings of discontinuity in the

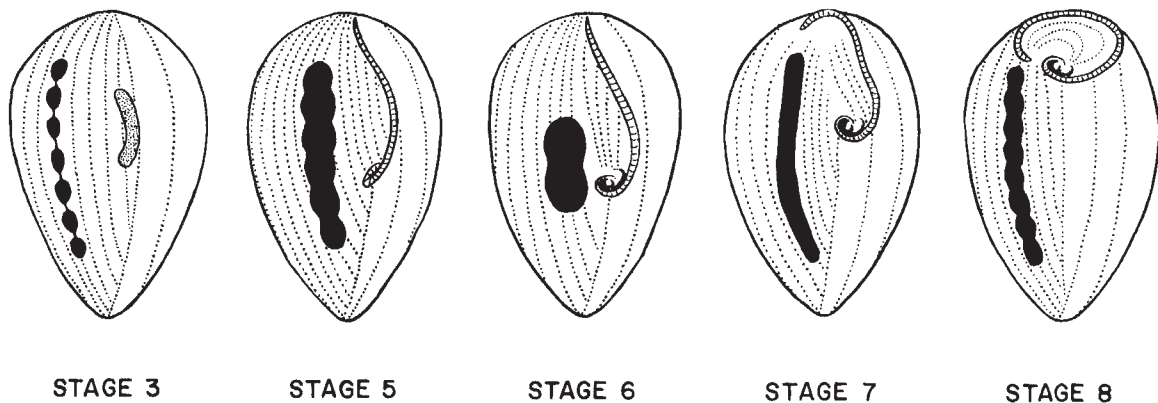


Fig. 1 Some stages of oral regeneration in *Stentor*. Stage 3, showing curved oral primordium (stippled); stage 5, nuclear coalescence; stage 6, gullet formation; stage 7, nuclear elongation; stage 8, nuclear nodulation.

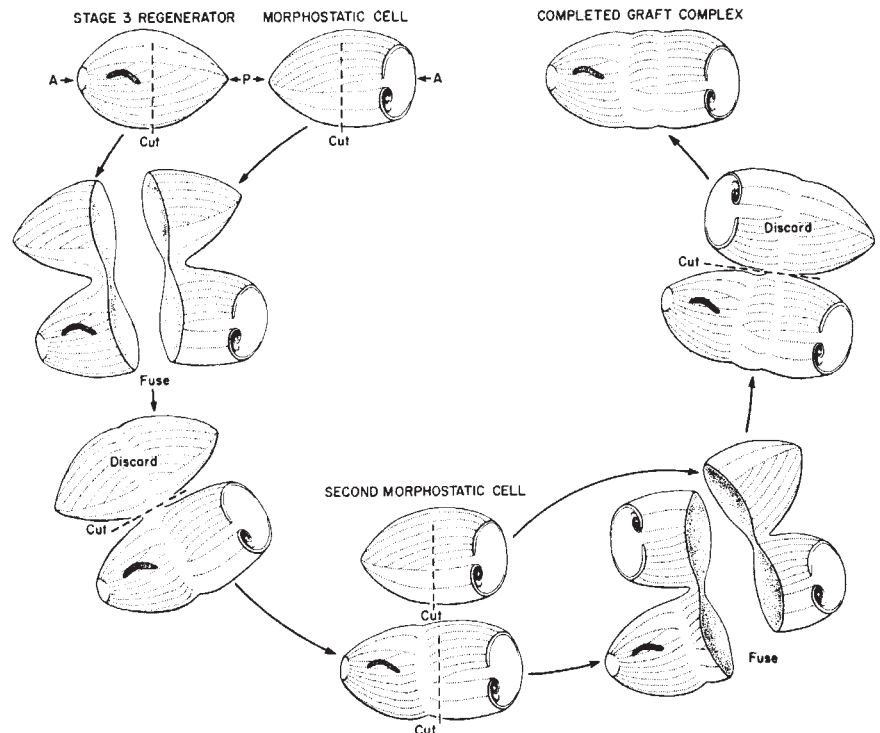
Here I report some experiments with *Stentor* which suggest a possible mechanism for the generation of such nuclear differences in morphology and synthesis. Previous experiments on the role of the cell surface in generating asynchrony of nuclear morphology^{8,9} have now been extended; the results indicate that nuclei with asynchronous morphology are also asynchronous with respect to DNA synthesis.

Experimental system

Stentor is a large (1 mm) ciliate which is one of the classic organisms for studies involving cell microsurgery. The most prominent feature of cell division is the development of the oral primordium, which gives rise to the feeding structures of the posterior daughter cell by production of kinetosomes (basal bodies) and associated structures at the junction of wide and

striped cortical surface layer (Fig. 3) which heals slowly over a period of 3–4 h. Although the cortical membrane heals with time, breaks in the cortical fibrillar structures (kinetodesmal fibres, and myonemes) do not heal. When the food vacuoles of one graft component were labelled with small plastic spheres before the operation, the spheres became evenly distributed throughout the endoplasm of the entire graft complex within 10–15 min⁸; the two members of the graft complex therefore share a common endoplasm from the time of the operation. The fact that cortical discontinuities were usually accompanied by nuclear asynchrony although the endoplasm mix freely suggested that nuclear morphology might be controlled by cell surface changes rather than by diffusion of a regulatory substance through the endoplasm from the regenerating component to the morphostatic one. Since DNA synthesis normally stops at the time of macronuclear coalescence, it seemed

Fig. 2 Procedure used in making graft complexes. The anterior half of a cell in stage 3 of regeneration is grafted to a morphostatic stentor in heteropolar orientation. A cut is then made in the morphostatic stentor just below the line of heal and the anterior half of a second morphostatic cell is added in homopolar orientation with respect to the first one. Most of the first morphostatic cell is removed during the operation so that it persists only as a thin disk separating the graft components.



interesting to examine graft complexes with asynchronous nuclear morphology for gross asynchrony of DNA synthesis.

Induced asynchrony of macronuclear DNA synthesis

S. coeruleus (Stella strain, obtained from Dr Vance Tartar) was cultured in a modified Peters' solution; the food organism was *Tetrahymena pyriformis* grown on *Aerobacter aerogenes*. The cultures, kept in Petri dishes, were fed *ad libitum* from 1000 to 1800, starved overnight and used the following day. This schedule generally produced cultures that were well enough fed for most cells to be synthesising DNA but starved long enough to clear the endoplasm of most food vacuoles. This proved necessary to permit observation of nuclear morphology and facilitate microsurgery, which was carried out free-hand with a glass needle. DNA synthesis was assessed by transferring graft complexes to Peters' medium containing ^3H -thymidine (New England Nuclear or Amersham Searle, 17–20 $\mu\text{Ci mmol}^{-1}$, 50 $\mu\text{Ci ml}^{-1}$) for 30 min. Squash preparations were processed for autoradiography with Kodak NTB3 emulsion as described previously¹¹. Regeneration was induced by a brief treatment with 10% sucrose, which removes the band of adoral membranelles from the oral apparatus and initiates oral primordium formation.

Preliminary studies confirmed previous work on dividing¹¹ and regenerating¹³ stentors; silver grains were not found over the coalesced or elongating nuclei of regenerating stentors, although morphostatic cells and cells in earlier stages of regeneration taken from the same culture showed heavy nuclear label. The absence of label at stages 6 and 7 is not caused by failure of the cells to take up ^3H -thymidine since coalesced and elongating nuclei are unlabelled even when the isotope is injected¹³.

Graft complexes with regenerating (stage 3) and morphostatic components separated by cortical discontinuities were prepared in groups by grafting for 1 h; they were then maintained in Peters' medium until nuclear coalescence had occurred in the regenerating component 2–3 h later. They were transferred to Peters' medium containing ^3H -thymidine, squashed on slides in a known orientation and prepared for autoradiography. The resulting autoradiographs were examined for gross differences in labelling. The differences were so great

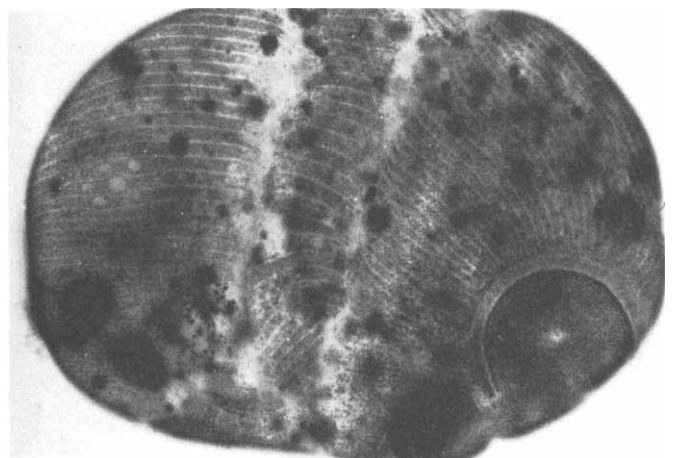
that only a few graft complexes were difficult to evaluate visually and these were discarded along with unlabelled graft complexes.

Ninety-six per cent of the 52 graft complexes examined were grossly asynchronous with respect to DNA synthesis (Fig. 4a); a typical example is shown in Fig. 5. The chain of nodes was always more heavily labelled than the coalesced nucleus.

Nuclear coalescence was induced in some graft complexes with cortical discontinuities, presumably because cortical healing sometimes occurred rapidly enough to facilitate re-establishment of synchrony. In all of 39 such graft complexes examined, both coalesced nuclei were unlabelled (Fig. 4b).

To examine synchrony in graft complexes lacking cortical discontinuities, a large stentor in stage 1 of regeneration was grafted directly in heteropolar orientation to a morphostatic cell; the graft complexes were incubated in Peters' medium for 2–3 h, placed in ^3H -thymidine for 30 min and prepared for autoradiography. Synchrony of nuclear labelling was found in 99% of the 73 labelled graft complexes examined (Fig. 4c).

Fig. 3 Newly created graft complex, showing double rings of cortical discontinuity at graft boundaries.



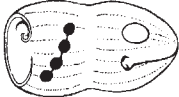
TYPE OF GRAFT COMPLEX	TOTAL NO. OF GRAFT COMPLEXES	DNA SYNTHESIS IN GRAFT COMPLEXES	
		Synchronous	Asynchronous
<i>a</i> 	52	2 (4%)	50 (96%)
<i>b</i> 	39	39 (100%)	—
<i>c</i> 	73	72 (99%)	1 (1%)
<i>d</i> 	17	2 (12%)	15 (88%)

Fig. 4 Summary of experiments on induced asynchrony of macronuclear DNA synthesis in *Stentor* graft complexes. *a*, Morphostatic stentor grafted to stentor in stage 3 of regeneration; graft components separated by cortical discontinuities. The regenerating component has reached stage 6 and nuclear morphology is asynchronous. *b*, Same as (*a*) except that nuclear morphology is synchronous presumably because of cortical healing. *c*, Morphostatic stentor grafted to stentor in stage 1 of regeneration; no cortical discontinuities. *d*, Morphostatic stentor grafted to stentor in stage 5; regenerating component has reached stage 6. Asynchronous nuclear morphology; no cortical discontinuities.

Since stentors selected at random from a given culture usually differ widely in extent of labelling, this strongly suggested that the graft complexes had become synchronised. Later experiments showed that a similar degree of synchrony occurred after 1 h in Peters' medium.

Since the central barrier ring does not interfere with the exchange of food vacuoles between the graft components, one would not expect it to impede the free exchange of ^3H -thymidine. But this was tested directly. *Stentor* takes up isotopes

primarily through the gullet and decapitated organisms accumulate little or no label¹⁴. Graft complexes with cortical discontinuities were prepared and the oral apparatus was excised from one graft component at the time of the operation. After 2–3 h in Peters' medium, the graft complexes were placed in ^3H -thymidine for 30 min and squashed in a known orientation. If the discontinuities had interfered with diffusion of ^3H -thymidine, one would expect the nucleus of the intact component to be more densely labelled but examination of

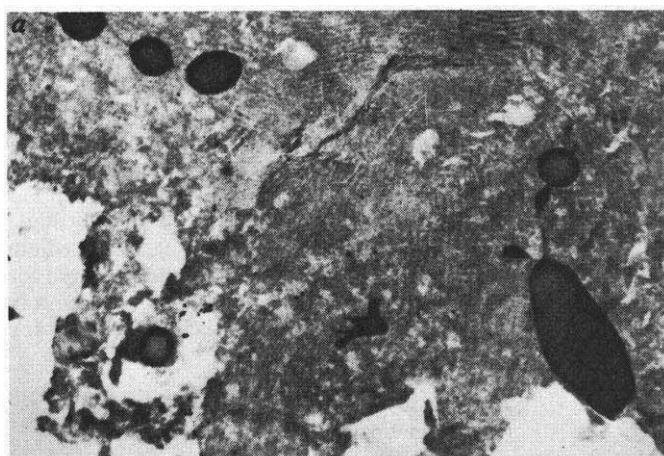
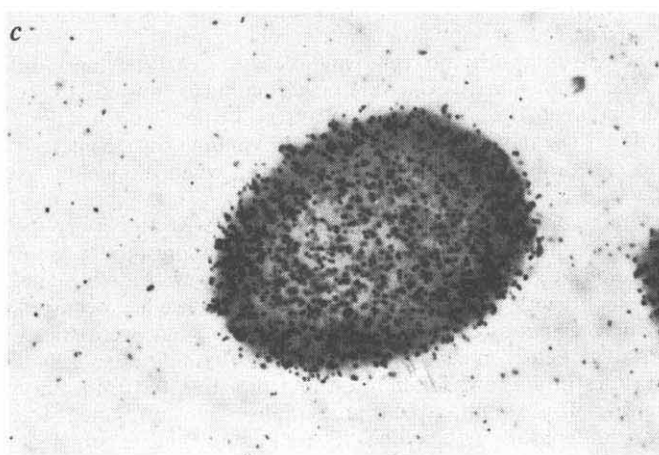
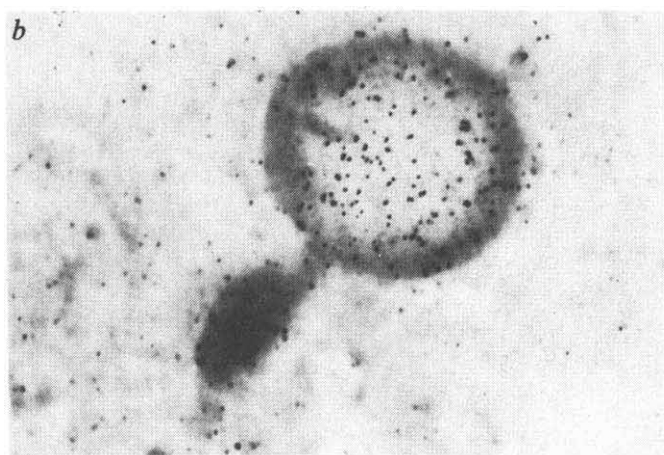


Fig. 5 Macronuclear DNA synthesis in graft complex with differential nuclear morphology. *a*, Graft complex, with coalesced nucleus of regenerating component (right) and three nuclear nodes of morphostatic component (left) ($\times 140$). *b*, Autoradiograph, part of the coalesced nucleus shown in (*a*) ($\times 880$). *c*, Autoradiograph, one node of the chain-form nucleus shown in (*a*) ($\times 880$).



32 such graft complexes showed that when differences in extent of labelling occurred they were random.

A final experiment was designed to determine whether the asynchrony of DNA synthesis in graft complexes with cortical discontinuities might be entirely or partly caused by the fact that one nucleus was in the chain form and the other was coalesced. This seemed likely because a coalesced nucleus transferred for 2–3 h to an S phase stentor resumes DNA synthesis but is less densely labelled than the chain nucleus of the host cell⁷. Coalesced nuclei, however, might behave differently in graft complexes containing their own cytoplasm as well as interphase cytoplasm; DNA synthesis was therefore examined in graft complexes containing half of a morphostatic stentor and half of a stentor in stage 5 or 6. In such grafts, the nucleus of the morphostatic component is not induced to coalesce but persists as a chain of nodes while the nucleus of the differentiating component is coalesced. Groups of such graft complexes, prepared over a 30-min period, were incubated in Peters' medium for 1 or 2 h, transferred to ³H-thymidine for 30 min and prepared for autoradiography. In 59% of the 17 labelled graft complexes examined, the coalesced nucleus was labelled, indicating that coalesced nuclei can be induced to resume DNA synthesis when they are part of a stage 6-morphostatic graft. Eighty-eight per cent of the graft complexes showed gross asynchrony of labelling with the coalesced nucleus always less heavily labelled than the chain of nodes (Fig. 4d). These results suggest that the gross asynchrony of DNA synthesis in graft complexes with cortical discontinuities is a consequence of the fact that coalesced nuclei synthesise less DNA than chain-form nuclei when sharing a common cytoplasm. The cortex therefore seems likely to regulate macronuclear DNA synthesis through some structural alteration associated with nuclear coalescence (such as a possible change in the structural state of chromatin and/or the macronuclear membrane).

Cortical control of nuclear asynchrony

It is clear from the experiments described here that identical nuclei sharing a common cytoplasm can be made grossly asynchronous with regard to morphology and DNA synthesis if they are associated with separate regions of cell surface. In *Stentor*, this association seems to involve physical attachment of the nucleus to a specific region of cortex¹⁰. Tartar's work¹⁰ has indicated that the cell surfaces of regenerating and morphostatic *Stentor* differ in ways which are important for

control of morphogenesis. My work provides further evidence for such differences and suggests their importance for control of nuclear morphology and synthesis. The finding that nuclear differences can be determined by cell surface differences is interesting in view of accumulating evidence that the ciliate cortex is regionally differentiated. In *Stentor*, the regions of wide and narrow cortical striping have different morphogenetic properties¹⁰; in *Paramecium*, cilia isolated from one mating type only adhere to cells of opposite mating type at specific cortical sites¹⁵. Most of these differences provide no clue as to how the cortex may control the behaviour of the nucleus. But the recent finding that the anterior and posterior regions of *Paramecium* respond differently to mechanical stimulation in terms of permeability to ions¹⁶ suggests at least one specific way in which differentiated regions of cell surface might control the behaviour of underlying nuclei. Finally, in ciliates, nuclear differentiation is only one of many phenomena involving highly localised asynchronous behaviour of apparently identical organelles sharing a common cytoplasm. In *Nassula*, for example, the pre-existing cytopharyngeal basket is resorbed during cell division while two new baskets are forming for the daughter cells¹⁷; during reorganisation in *Stentor*, the original gullet dedifferentiates as a new one develops nearby¹⁰. My findings may be relevant to some of these phenomena as well as to nuclear differentiation in other cell types.

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The vegetation of Tertiary islands on the Ninetyeast Ridge

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Pollen from deep-sea drilling sites on the Ninetyeast Ridge which reflects island floras that flourished in the Palaeocene and Oligocene, shows pronounced similarity to Australian and New Zealand early Tertiary floras. Although they were closer to southern landmasses during the Tertiary, these islands were truly oceanic, and their colonisation occurred through long-distance dispersal mechanisms.

DEEP-SEA drilling on the Ninetyeast Ridge has shown the presence of shallow-water sediments at several points along the ridge crest^{1,2}. At Deep Sea Drilling Project (DSDP) sites 214 and 254, in the northern and southern sectors of the ridge respectively (Fig. 1), sequences of volcanoclastics, sands, clays, pebble conglomerates, and organic-rich intervals lie immediately above the oceanic basement and show evidence of subaerial weathering and deposition. Among the strongest evidence for subaerial conditions is the presence of well preserved spores and pollen, reflecting the existence of a land vegetation of some diversity at points

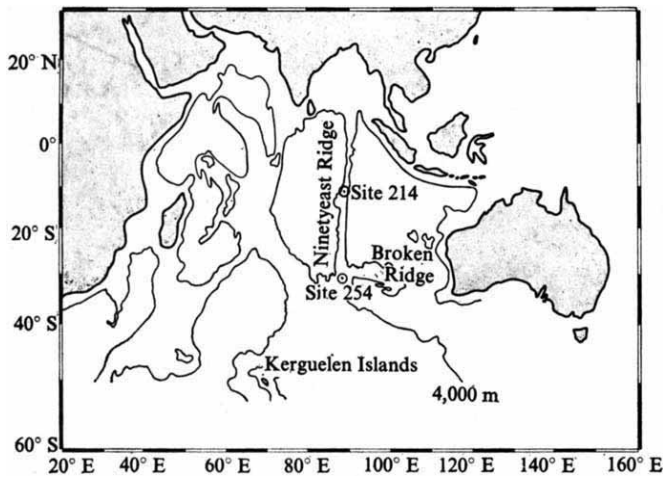


Fig. 1 Sketch map of the Indian Ocean showing the Ninetyeast Ridge and related features, and the position of Deep Sea Drilling Project sites 214 and 254.

along the ridge crest during the Palaeogene. We interpret such vegetation as that of oceanic islands that were emergent along the ridge from perhaps the latest Cretaceous to the late Oligocene; present fossil evidence establishes the presence of such vegetated land areas during the Palaeocene and Oligocene.

Preliminary accounts of the microfloras have already been published^{3,4}; this paper documents the occurrence of these pollen-rich sediments, discusses the floristic composition of the vegetation they reflect, the dispersal problems faced by the island floras, and the influence on them of former distributions of land and sea areas. The detailed taxonomical classification of dispersed spores and pollen is currently being undertaken (Kemp and Harris, unpublished).

Tectonic setting of the Ninetyeast Ridge

The Ninetyeast Ridge extends for some 5,000 km along the 90°E meridian, from 32°S to at least 9°N. The ridge crest deepens progressively from south to north, and sediments immediately above oceanic basement at crest drilling sites increase in age northwards. The nature of sedimentary sequences at the drilling sites accord with a northward subsidence of the ridge throughout its history⁵.

The ridge has been interpreted^{6,7} as having formed from an extrusive pile accumulating at the junction of a former active spreading centre and a north-south trending transform fault, parallel to the present ridge. According to that model, volcanism was associated with motion along a transform fault between the Indian and Australian plates until their fusion in the late Oligocene. The formation of both the ridge and the attached Indian plate in relatively high southern latitudes and their subsequent northward movement is supported by magnetic anomaly data⁸, by the palaeomagnetism of basalts at drilling sites⁹, and by calcareous microfossils indicating warming of depositional conditions in younger strata¹⁰.

Formerly emergent areas on the ridge crest are likely to have been relatively small islands, as the configuration of the ridge places limits on their east-west dimension, and the age differences of basal sediments from north to south precludes the emergence of large areas at any one time.

Stratigraphy of sampled sites

The generalised stratigraphy of sites 214 and 254 is shown in Fig. 2. At site 214, well preserved spores and pollen were

recovered from interbedded volcanoclastics and lignites; this sequence is non-marine. These assemblages continue in to overlying glauconitic carbonate silts and sands which contain dinoflagellate cysts, acritarchs, foraminifera, nannofossils and molluscs. Foraminifera in the glauconitic unit indicate a Palaeocene age—P4 (ref. 11); the nannofossil zone of *Heliolithus kleinpellii* is present at 337 m below the

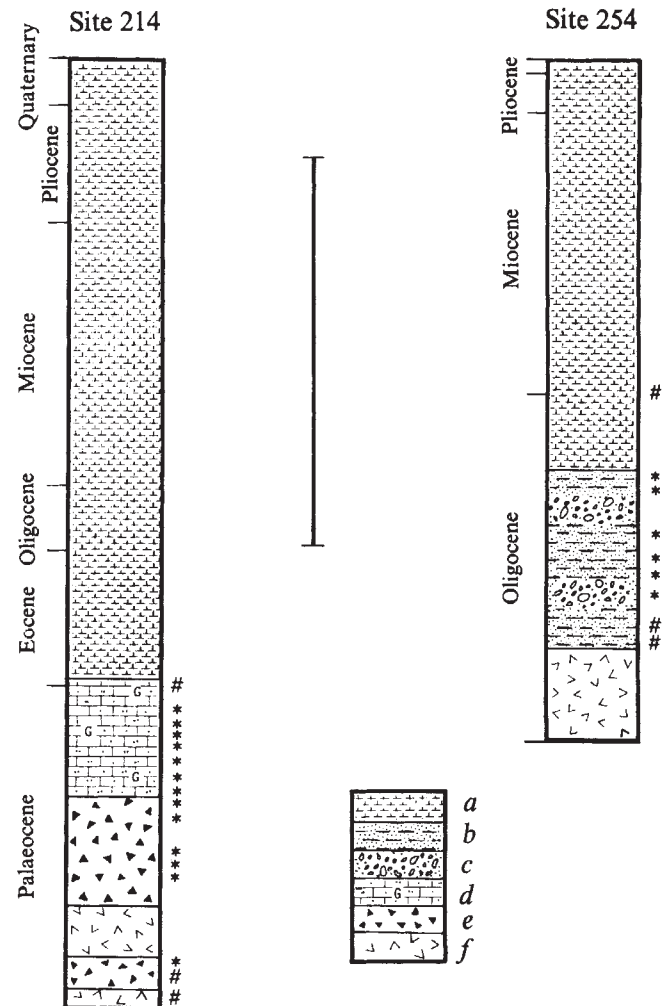


Fig. 2 Simplified stratigraphic columns at sites 214 and 254, with the position of spore and pollen-bearing core samples shown on the right of the columns. Modified from refs 10 and 12. Scale bar, 200 m; site 214, depth of penetration 500 m starting at a water depth of 1,665 m; site 254, depth of penetration 343.5 m starting at a water depth 1,253 m. *, Productive samples; †, barren samples; a, calcareous ooze; b, sandy and silty clay; c, pebble conglomerate; d, glauconitic carbonate, silt and sand; e, volcanoclastics interbedded with lignites; f, basalt.

sea floor, and the section may be as old as the *Cyclococcolithina robusta* zone at 389 m (ref. 12). This mid-Palaeocene date has been extrapolated down into the volcanoclastic sequence below as the spore and pollen assemblages are essentially the same throughout.

At site 254, excellently preserved spores and pollen occur abundantly in the lowest sedimentary unit. This consists of poorly sorted sandstone and clay, some pebble conglomerates, has a high content of organic matter, and grades downwards into weathered olivine basalt. It possibly originates from the subaerial weathering of a basaltic terrain, with rapid deposition of weathering products in a quiet, shallow marine environment¹⁰; a lagoonal setting adjacent to vegetated highlands is perhaps represented. Age estimates of the palyniferous basal unit are ambiguous;

Table 1 Palynological form species of angiosperm and gymnosperm pollen recovered from sites 214 and 215, Ninetyeast Ridge, with their probable botanical affinity

Palynological form species	Probable botanical affinity	DSDP site	
		214	254
Angiosperms*			
<i>Arecipites</i> cf. <i>waitakiensis</i> (McIntyre)	Palmae		X†
<i>Arecipites</i> sp.	Palmae	X	
<i>Australopolis obscurus</i> (Harris)	Unknown	X	
<i>Clavatipollenites hughesii</i> Couper	Chloranthaceae (<i>Ascarina</i> ?)	X	X
<i>Cupanieidites orthoichus</i> Cookson and Pike	Sapindaceae (Cupanieae)		X
<i>Echiperiporites</i> sp.	Unknown		X
<i>Gothanipollis</i> cf. <i>gothani</i> Krutzsch	Loranthaceae		X
<i>Halorgacidites</i> cf. <i>harrisii</i> (Couper)	Casuarinaceae (<i>Casuarina</i>)	X	X
<i>Milfordia homeopunctata</i> (McIntyre)	Restionaceae (<i>Restio</i> ?)		X
<i>Myrtacidites</i> cf. <i>mesonesus</i> Cookson and Pike	Myrtaceae		X
<i>Myrtacidites</i> spp.	Myrtaceae	X	X
<i>Proteacidites</i> cf. <i>symphonemoides</i> Cookson	Proteaceae		X
<i>Proteacidites</i> sp.	Proteaceae	X	
<i>Nothofagidites</i> spp.	Fagaceae (<i>Nothofagus</i>)	X	X
<i>Psilotricolporites</i> sp.	Unknown		X
<i>Psilodiporites</i> cf. <i>redundantis</i> Guzman	Moraceae/Urticaceae		X
<i>Polycolpites</i> sp.	Unknown		X
<i>Sapotaceoidapollenites rotundus</i> Harris	Sapotaceae		X
<i>Sparganiaceapollenites</i> sp.	Sparganiaceae		X
<i>Spinizonocolpites prominatus</i> (McIntyre)	Palmae		X
<i>Schizocolpus marlinensis</i> Stover	Didymelaceae (<i>Didymeles</i> ?)	X	X
<i>Striatricolporites</i> sp.	Unknown		X
<i>Tricolpites reticulatus</i> Cookson	Gunneraceae (<i>Gunnera</i>)	X	X
<i>Tricolpites</i> spp.	Unknown	X	X
<i>Tricolporites</i> spp.	Unknown	X	X
<i>Tubulifloridites</i> cf. <i>antipodica</i> Cookson	Compositae (Tubuliflorae)		X
<i>Tubulifloridites</i> sp.	Compositae?	X	
Gymnosperms*			
<i>Araucariacites australis</i> Cookson	Araucariaceae (<i>Araucaria</i> ?)		X
<i>Lygistipollenites florinii</i> (Cookson and Pike)	Podocarpaceae (cf. <i>Dacrydium</i>)	X	
<i>Microcachrydites antarcticus</i> Cookson	Podocarpaceae (cf. <i>Microachrys</i>)	X	X
<i>Podocarpidites</i> spp.	Podocarpaceae	X	X

*Only previously published form species are listed; new species are assigned to form genera only.

†X denotes presence.

the firmest control comes from the lower part of the overlying calcareous ooze unit, which has yielded middle to late Oligocene planktonic foraminifera¹⁰. Below that, foraminifera, ostracods and molluscs are not stratigraphically diagnostic, and an age range of Eocene to late Oligocene has been suggested¹⁰.

Composition of the microfloras

Form species of angiospermous and gymnospermous origin present at the two sites are listed in Table 1. At site 214, the major pollen groups include 15 form species of angiosperms, 18 pteridophytes, 4 conifers and 3 lycopods. Palm pollens are dominant, and pollen similar to that of the extant New Zealand and New Caledonian genus *Ascarina* is abundant. Pollen of *Gunnera* type is consistently present. Several species occur so rarely that a wind-blown origin cannot be discounted; included here are *Nothofagus* (*brassi* type), Proteaceae, Casuarinaceae and Didymelaceae grain types.

The flora represented at site 254 was more diverse, with, as minimum figures, some 26 angiosperms, 3 conifers, 17 ferns, 2 lycopods and a variety of fungi. Tricolporate pollens of indeterminate affinity are most abundant. Among pollen groups identifiable to family level, Myrtaceae is most common. Two types were identified; one is broadly similar to that found in *Lophomyrtus*, *Rhodomyrtus* and related taxa; the other resembles no known living form. Palm types are again abundant; generic affinities are obscure, but similarities to *Cocos*, *Jessenia* and *Paralinospadix* are evident. Moraceae/Urticaceae pollens are a prominent element, and Casuarinaceae grains are present in quantities which suggest they are indigenous rather than wind-blown. Proteaceae and Restionaceae are both represented by types known in the Australian Tertiary. Families of global distribution are represented by Compositae and

Graminae, both rare, and by Loranthaceae and Sapotaceae.

Conifer pollen at both sites suggests a largely podocarpaceous parentage. At site 254, coniferaean pollen makes up some 20% of the pollen spectrum. Araucariaceae types are common at this site, and have been tentatively identified as *Araucaria*. The fern spore spectrum at both sites is similar, and is dominated by cyatheaceous types, but Polypodiaceae, Gleicheniaceae and Schizaeaceae have been identified. Lycopod spores are rare, but three form species have been isolated. Of the abundant fungal remains at site 254, only fructifications of Microthyriaceae could be firmly identified.

Phytogeographical considerations

The plant microfossil assemblages indicate that the vegetation of these islands had much in common with the early Tertiary vegetation of Australia, New Zealand, southernmost South America and Antarctica. Few species occurred in common with Tertiary floras of India and Indo-Malaysian archipelago. Of 41 form-species of spores isolated at site 214, 28 have been identified, or have closely similar counterparts, in the Australian Tertiary. At site 254, 28 of the known 48 form species occur in the Australian Tertiary, and 19 are known from the Tertiary of New Zealand. Resemblances occur within all plant groups. Among conifers, the mixture of podocarpaceous and araucarian elements is typical of the Southern Hemisphere early Tertiary. Among angiosperms, species lists indicate that many common forms existed between continent and island, but qualitative differences lie in the extreme rarity of *Nothofagus* pollen at the island sites, and in the poor representation of Proteaceae.

Reconstructions of the Indian Ocean for the mid-Palaeocene and late Oligocene (Fig. 3) indicate that distances of the Ninetyeast Ridge islands from the southern

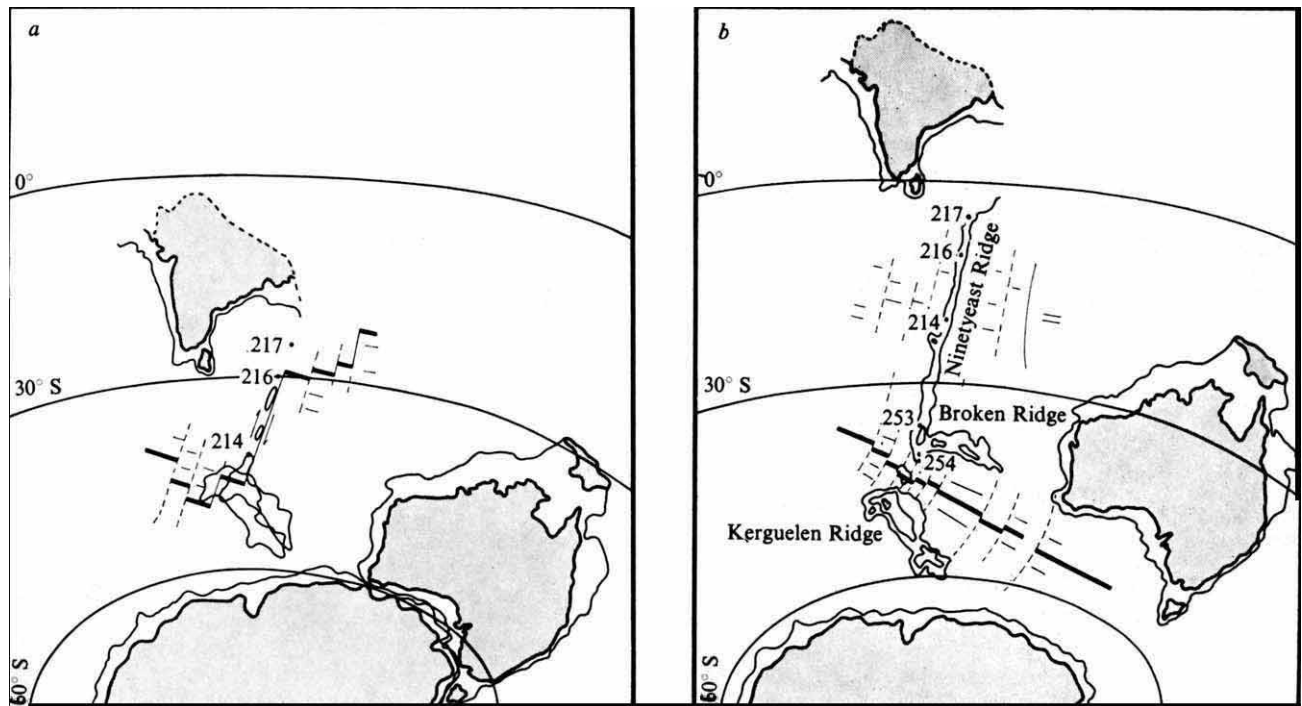


Fig. 3 Reconstructions of the eastern Indian Ocean for the mid-Palaeocene (a) and the late Oligocene (b), modified from ref. 7. Position of all Deep Sea Drilling Project drilling sites on the ridge crest is shown. Active centres of seafloor spreading are shown by heavy lines. Basalts at site 214 were being extruded approximately 60 Myr BP (a), along a transform parallel to the trend of the Ninetyeast Ridge; the Kerguelen and Broken Ridges are shown joined. At 25 Myr BP (b), when site 254 was emergent, a new axis of spreading, trending north-west separated Australia and Antarctica and Broken Ridge and Kerguelen. Site 214 has been spread to the north and submerged.

continents were much less in the early Tertiary in comparison with present geography, which must account in part for the similarity of the island floras with those of the landmasses to the south-east Islands which may have been emergent in the late Cretaceous would have been even closer to the continents. It does, however, seem likely that the sites were still truly oceanic, separated from the nearest continents by distances probably in excess of 1,000 km. The floras, therefore, must have become established through the long-distance dispersal of propagules, as has the vegetation of modern south temperate and subantarctic islands. Possible intermediate island sites, acting as biotic 'stepping-stones' between the southern continents and the ridge islands, may have been present along the formerly joined Broken and Kerguelen Ridges; beach gravels in a drilling site on Broken Ridge confirm that parts of it were emergent during the early Tertiary², and the Kerguelen islands have possibly been emergent since the Oligocene¹³.

Problems in understanding the colonisation of the Ninetyeast Ridge islands relate to the apparent inability of some of the modern families represented to cross extended water gaps. Adaptations for long-distance wind and water transport are unknown in Casuarinaceae and Myrtaceae, and the present disjunct distributions of these and other southern families such as Proteaceae and Restionaceae are usually explained by invoking former land connections. *Araucaria*, too, has a modern distribution in the Pacific accountable in terms of previous land connections¹⁴. Migration pathways to the early Tertiary *Araucaria* stands in the Indian Ocean, on Kerguelen^{15,16} and on the islands on the Ninetyeast Ridge, however, are more difficult to trace, and there are no Tertiary records from countries bordering that ocean. Podocarpaceae is another family poorly represented today on oceanic islands, although migration may have occurred through Indo-Malayan archipelagic islands during the late Tertiary.

Direct transport by winds and currents, and transport by biological agents are facilitated in regions of intense oceanic

and atmospheric circulation, and in this context it should be noted that the islands of the Ninetyeast Ridge, when emergent, lay in the belt of circumpolar circulation. The intensity of this circulation must have varied throughout the Palaeogene; in the Palaeocene, with Australia and Antarctica still joined, and in the absence of a polar ice cap, circulation may have been relatively sluggish. By the Oligocene, however, in the wake of the opening of a seaway between Australia and Antarctica, and the development of an Antarctic icecap¹⁷, strong westerly winds and currents probably prevailed.

Bird transport may have been significant in the establishment of many taxa on the Ninetyeast Ridge and Kerguelen sites. Birds must account for much seed transport to south temperate and subantarctic islands, although there have been few direct observations. Among the Ninetyeast Ridge microfloras there are some groups in which bird dispersal of seed is known—*Gunnera*, for instance¹⁸, and members of the Loranthaceae are known to be dispersed by birds, and transport of *Podocarpus* fruits by frugivorous birds has been suggested¹⁹, although the efficiency of such a dispersal mechanism over long ocean distances remains speculative. Moraceae, too, is a group with a high potential for this kind of dispersal and a possible example of the establishment of Myrtaceae (*Metrosideros*) on New Zealand shelf islands by migrating birds has been documented²⁰.

Comparison with modern island floras

The reconstructions of Fig. 3 suggest that the islands of the Ninetyeast Ridge, when emergent, lay somewhat south of 40°S. At present, in the eastern Indian Ocean, Amsterdam Island and St Paul Island lie at 37°55'S and 38°44'S, and Kerguelen Island lies at 49°30'S. The floras of these modern islands have few species; St Paul Island and Amsterdam Island at present support some 18 species of flowering plants^{21,22}, a figure somewhat lower than the 26 reflected in the pollen spectrum for site 254, which probably lay even farther south. Kerguelen Island supports about

21 flowering plant species. Modern island floras are further distinguished by a high ratio of pteridophytes to angiosperms—the occurrence of ‘fern bush’ communities on the more temperate of the southern oceanic islands, including Amsterdam Island, is a common feature²². On spore evidence, the pteridophyte assembly of the fossil islands seems comparable with those of the modern ones.

Even more striking than the apparently high diversity shown by the Tertiary island floras are the basic differences in floristic composition between them and the modern islands. Plants from high southern latitudes have been divided into “insular” and “continental” groups²³; in the former category are taxa with a widespread distribution on southern oceanic islands; the latter include groups at present largely confined to the continents—the families Centrolepidaceae, Proteaceae, Restionaceae, Myrtaceae, and the genus *Nothofagus* have been cited as being chiefly of that distribution. Those groups are absent from modern islands in the southern Indian Ocean in spite of the existence of apparently suitable habitats on some of them. Such an absence has been taken by biogeographers to indicate a lack of dispersal ability.

The fossil evidence to hand suggests that members of some of these ‘continental’ families did in fact become successfully established on islands on the Ninetyeast Ridge, where pollen of Myrtaceae, Restionaceae, Proteaceae, and that of Podocarpaceae and Araucariaceae occurs in quantities that are not suggestive of a wind-blown origin. Geological considerations dictate that establishment at these sites must have involved dispersal across significant ocean

gaps, so that these groups may not always have lacked vagility. It further suggests that their current absence from southern oceanic islands reflects the recent environmental history of those islands, all of which have been subjected, to a greater or lesser degree, to late Neogene chilling.

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letters to nature

Improved declination for transient X-ray source A0620—00

OBSERVATIONS with the Effelsberg 100-m telescope at a frequency of 4,600 MHz have resulted in an improved declination for the radio source believed to be associated with the transient X-ray source A0620—00 (ref. 1). The observations were made on August 24, 1975 with a cooled parametric amplifier of system temperature 65 K. The bandwidth was 200 MHz, and the half-power beamwidth of the telescope was 2.7'. The measurements were calibrated relative to NGC7027 and 3C286.

Twenty-one declination scans were made at right ascension (1950) 06 h 20 min 11.4 s, which was the interferometric position reported by Davis *et al.*². Each scan was 20' long, centred at declination (1950) —00°21'. The average of these scans is shown in Fig. 1. The baseline is not flat because of receiver instabilities (the system was operated without a Dicke switch), but the radio source is clearly present. The measured half-power width of 2.7' is that expected for a point source. The declination (1950) of the source is —00°19'36"±25". The peak antenna temperature was 0.037±0.008 K, which corresponds to a flux density of 28±6 mJy at 4,600 MHz.

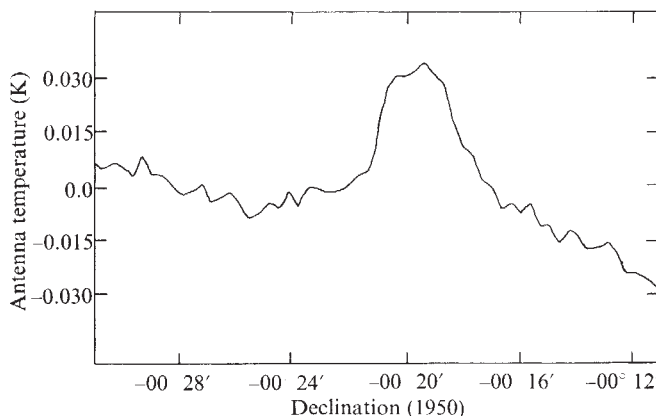
The declination measured here is consistent with the radio position reported by Craft³, the optical position of Boley and Wolfson³, and the X-ray positions given by Matilsky³ and by Eyles *et al.*². Our value for the flux density at 4,600 MHz is slightly larger than the 5,000-MHz value reported by Scott⁴ for an observation two days earlier than ours, although the numbers agree within the uncertainties. Davies⁵ reports that the flux density at 962 MHz was 21±9 mJy at the same epoch as our

observation. The corresponding spectral index between 962 and 4,600 MHz is therefore

$$\alpha = 0.18^{+0.49}_{-0.38}$$

where $S(\nu) \propto \nu^\alpha$. This value suggests that the spectrum at cm wavelengths is relatively flat, in agreement with the flux densities measured by Owen *et al.*⁴ at 1,418 and 2,695 MHz.

Fig. 1 Average of 21 declination scans made at RA (1950) = 06 h 20 min 11.4 s at 0850 UT, August 24, 1975. The half-power beamwidth of the telescope is 2.7' at the observing frequency of 4.6 GHz. The peak source antenna temperature of 0.037±0.008 K corresponds to a flux density of 28±6 mJy for a point source.



We scanned this position again on both September 7 and 8, 1975 but failed to detect the radio source. The data were of poor quality, but at that time we estimate that the flux density at 4,600 MHz was less than 20 mJy.

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¹ IAU Circ. No. 2817.

² IAU Circ. No. 2822.

³ IAU Circ. No. 2819.

⁴ IAU Circ. No. 2823.

⁵ Davis, R. J., Edwards, M. R., Morison, I., and Spencer, R. E., *Nature*, 257, 659 (1975).

X-ray sources and planetary nebulae

CURRENT models of X-ray sources in binary systems generally assume accretion on to a neutron star, a black hole or a white dwarf as the mechanism of X-ray production. The accretion takes place from either a stellar wind or the inner Lagrangian point¹⁻⁵. Here we explore the possible existence of a compact X-ray source in a binary system, where the companion star ejects its envelope to become a planetary nebula.

Consider first the possibility that the compact object is a neutron star. The accretion rate from a wind is given approximately by^{6,7}:

$$\dot{M}_{acc} = 1.8 \times 10^{19} \left(\frac{M_x}{M_\odot} \right)^2 \left(\frac{\dot{M}_{pn}}{10^{21} \text{ g s}^{-1}} \right) \left(\frac{a}{10^{15} \text{ cm}} \right) \times \left(\frac{V_p}{10^6 \text{ cm s}^{-1}} \right) \left(\frac{\bar{V}}{10^6 \text{ cm s}^{-1}} \right)^{-3} \text{ g s}^{-1} \quad (1)$$

where M_x is the mass of the accreting star, $\dot{M}_{pn}$ the rate of mass ejection, a the separation between the stars, V the velocity of the wind and $\bar{V} = (V_r^2 + V_{sound}^2)^{1/2}$ where V_r is the relative velocity between the accreting star and the wind. Assuming typical values for the mass of the ejecting star $\sim 1.3M_\odot$, $a \sim 10^{15}$ cm, $\dot{M}_{pn} \sim 10^{21}$ g s⁻¹, $V_p \sim 3 \times 10^6$ cm s⁻¹, we find: $\dot{M}_{acc} \sim 8 \times 10^{16}$ g s⁻¹ which is in the range of typical ($\sim 10^{17}$ g s⁻¹) accretion rates for compact X-ray sources. Thus, the X-ray luminosity of the proposed source is comparable to known X-ray source luminosities. The lifetime of such a source will be of the order of the ejection time $\sim 10^2$ – 10^4 yr, unless the planetary phenomenon is a recurrent one. (Observations of FG Sag (ref. 8) imply that the planetary phenomenon may be a recurrent one.)

The question arises, however, whether a binary system in which one component is a neutron star and the other ejects a planetary nebula is a possible consequence of stellar evolution. We shall distinguish here a few possibilities.

For massive close binaries (A) stellar evolution estimates show⁹ that a neutron star can be formed via a supernova explosion if the mass of the primary is greater than $16M_\odot$. This high mass is dictated by severe mass transfer during evolution. Stars with lower mass probably evolve into white dwarfs.

Consider such a system with masses of $16M_\odot$ and $2M_\odot$. The mass transfer process, which eventually occurs in such systems, lasts a few thousand years and yields a final primary mass of the order of¹⁰:

$$\frac{M_f}{M_\odot} \sim 10^{-0.96} \left(\frac{M_i}{M_\odot} \right)^{1.4} \quad (2)$$

For $M_i \sim 16M_\odot$, $M_f \sim 5.3M_\odot$ (we assume a loss of $\sim 1M_\odot$ during the transfer process). A helium star with $5.3M_\odot$ evolves

in $\sim 10^6$ yr, and ends its evolution as a supernova. This time is too short for any appreciable evolution of the secondary star.

Present theories of planetary formation do not consider stars as massive as our companion star ($\sim 11.7M_\odot$) as possible progenitors of planetary nebulae (however, since these stars are so rare compared with stars of $\sim 1.4M_\odot$ it is impossible to exclude the possibility of their becoming planetary nebulae on observational grounds). One would, therefore, like to see whether it is possible for the supernova ejecta to blow off most of the mass of the second star. The answer depends on the energy required and the efficiency of the process.

The binding energy of the outer layer is:

$$E_b \sim 3.8 \times 10^{48} \left(\frac{M}{M_\odot} \right)^2 \left(\frac{R}{R_\odot} \right)^{-1} \text{ erg} \quad (3)$$

where M is the mass of the outer layer and R the radius of the star.

The energy needed to separate the two stars to the distance required by the correct accretion rate is approximately:

$$E_s \sim 9.1 \times 10^{47} \left(\frac{M_1'}{M_\odot} \right) \left(\frac{M_2'}{M_\odot} \right) P^{-2/3} \left(\frac{M_1 + M_2}{M_\odot} \right)^{-1/3} \text{ erg} \quad (4)$$

where P is the period (in days) before separation; M_1 , M_2 the masses before explosion; M_1' , M_2' the masses after explosion. On the other hand, the available kinetic energy going into the cone spanned by the second star is:

$$E_a \sim 1.4 \times 10^{49} \left(\frac{M_{ej}}{M_\odot} \right) \left(\frac{V_{ej}}{10^9 \text{ cm s}^{-1}} \right)^2 \left(\frac{R_2}{R_\odot} \right)^2 \times \left(\frac{M_1 + M_2}{M_\odot} \right)^{-2/3} \left(\frac{P}{1 \text{ d}} \right)^{-4/3} \text{ erg} \quad (5)$$

Putting in some typical values: $M \sim 10M_\odot$, $R \sim 5R_\odot$, one obtains $E_b \sim 7.6 \times 10^{49}$ erg (this should be reduced since the outer envelope is not so tightly bound). For $M_r + M_1 \sim 17M_\odot$, $M_1' \sim M_\odot$, $M_2' \sim 1.3M_\odot$, $P \sim 1$ d, we find $E_s \sim 4.6 \times 10^{47}$ erg. Taking: $M_{ej} \sim 4M_\odot$, $V_{ej} \sim 0.5 \times 10^9$ cm s⁻¹, $R_2 \sim 5R_\odot$, $M_1 + M_2 \sim 17M_\odot$ and $P \sim 1$ d, we get: $E_a \sim 5.3 \times 10^{49}$ erg. This seems energetically possible, but detailed calculations show that the efficiency of this process is very low (E.E. Salpeter, personal communication). Consequently, if we start with a close binary and neutron stars are formed only by supernova explosions, and only relatively low mass stars are capable of ejecting a planetary nebula, then the probability to form such an X-ray source is small.

For low mass widely separated binaries ($a \sim 10^{15}$ cm, called case B) a different type of evolution is obtained. In this case the minimum mass for producing a neutron star is $\sim 4M_\odot$ whereas the secondary can have initially $M \sim 1.2M_\odot$.

Another possibility, to be called case C, is to assume the accreting compact X-ray source to be a white dwarf^{11,12}. (The problem of the production of the X rays can be overcome since an X-ray tail is obtained from an accreting white dwarf, similar to the γ -ray tail found in neutron stars (S. L. Shapiro and E.E. Salpeter, unpublished)). Clearly no supernova phenomenon is called for here. The accretion rate required to provide the observed luminosities (10^{36} – 10^{37} erg s⁻¹) demands a separation of at most 5×10^{13} cm. The emission, however, will be mostly in the ultraviolet with some X-ray emission.

Assuming that X-ray sources of the types described, exist in our Galaxy, we evaluate the probability of observation for each case.

For A, a neutron star can be formed if $M_{primary} \gtrsim 16M_\odot$. The estimate of the number of such binaries in the Galaxy (after mass transfer) is about 4,000 (ref. 9). The He star evolves in

Table 1 Possible coincidences between X-ray sources and planetary nebulae

Source	Name	Right ascension (h min s)	Declination (° ' ")	Area of X-ray error box (degree ²)	Comments
X-ray Planetary nebula	3U1735-28 Th 3-35	17 35 24 17 35 5	-28 27 0 -28 41	0.04	Known to be a transient source
X-ray Planetary nebula	3U1735-44 Fg 2	17 35 12 17 35 7	-44 25 12 -44 08	0.002	—
X-ray Planetary nebula	3U1714-39 H2-6	17 14 55 17 14 56	-39 18 00 -39 16 10	0.083	Nebula probably old
X-ray Planetary nebula	3U1746-37 H1-36	17 46 48 17 46 24	-37 00 36 -37 00 36	0.0184	Counterpart globular cluster NGC 6441?

$\sim 10^6$ yr whereas the secondary ($\sim 1.4M_{\odot}$) requires $\sim 10^9$ yr before ejecting its envelope. The ejection itself (and therefore the X-ray stage) lasts at most $\sim 10^4$ yr. Thus the probability of discovery is negligibly small.

In case B with $M_{\text{primary}} \gtrsim 4M_{\odot}$, $M_{\text{secondary}} \sim 1.2-1.4M_{\odot}$, there are $\lesssim 7 \times 10^4$ such binaries in our Galaxy¹³. Taking into account the relative lifetime of the X-ray phase compared to stellar evolution, we expect the number of such sources in the galaxy to be about one.

Finally in case C, we assume the X-ray source to be a white dwarf. The expected number¹³ of such sources is ~ 70 , (these numbers should of course be multiplied by the ratio of the accessible volume in X rays and visible to the total galactic volume).

Dust formation during the early stage of envelope expansion gives rise to infrared radiation. It is expected, therefore, that X-ray sources powered by accretion from a young planetary nebula should be infrared sources.

An interesting X-ray source which possibly coincides with an infrared and radio source is^{14,15} Cyg X-3. The small separation ($P = 4.8$ h) requires a low mass ejection rate to avoid total extinction of the source. For $\dot{M}_{\text{pn}} \sim 10^{19} \text{ g s}^{-1}$ we obtain $\dot{M}_{\text{acc}} \sim 3.8 \times 10^{20} \text{ g s}^{-1}$, which is too high, but regulation by the Eddington limit may reduce this value to the required one.

A search for possible coincidences between observed X-ray sources¹⁷ and observed planetary nebulae¹⁶ yielded a few candidates, the best of which are given in Table 1 with some comments.

To conclude, the probability of observing an X-ray source in the form of a compact object accreting from an expanding planetary nebula is sensitive to the nature of the compact object (black holes have not been discussed) and to the course of evolution. We expect in such systems a coincidence between the X-ray source (or ultraviolet source) and infrared sources (or very young planetaries). The end product of a planetary nebula and a white dwarf should be a double white dwarf binary, one such system—LDS275AB—is known¹⁸ (with 4'' separation and having $m_v = 15$, $M_v = 13$ corresponding roughly to a separation of $\sim 1.5 \times 10^{15}$ cm) and there are a few other possible candidates for such systems.

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Qualitative features of synchrotron radiation in an inhomogeneous magnetic field

A UNIFORM, large scale magnetic field is probably an abstraction not found in nature. All sorts of instabilities may arise, and a filamentary or sheet-like structure is most likely to ensue. For example, it is well known that a picture of the optical continuum of the Crab Nebula shows a cotton-like structure, on a scale much smaller than the wisps ($\sim 10^{16}$ cm).

If the magnetic field is non-uniform, the estimates of the lifetime of energetic electrons against synchrotron losses must be modified, whereas for the low energy radiation there occurs another phenomenon. This is most easily seen if we suppose that the magnetic field is confined to plane sheets of average thickness R and total volume V_0 . The magnetic field, H , is supposed to be constant in the sheets, and zero outside.

The total power radiated by the system of sheets is $L(v) = NV_{\text{eff}}(v)P(v)$, where N is the number of sheets, $V_{\text{eff}}(v)$ is the effective volume per sheet radiating at a given frequency, and $P(v)$ is the power at given frequency emitted per cm³. The point is that the effective volume depends on the energy of the electrons (which are supposed to be injected from the outside) and therefore on the frequency. In fact low energy electrons can penetrate into the sheets only up to a distance of the order of the cyclotron radius r_c .

The effective volume is therefore $V_{\text{eff}} = V_0 r_c / R$. Now, $r_c \propto \gamma \propto v^{1/2}$, where γ is the Lorentz factor. Therefore

$$L(v) \propto NV_0 v^{1/2} P(v) \quad (1)$$

The usual approximation has been used in which the synchrotron spectrum per electron is a delta function.

From the energy at which $V_0 = V_{\text{eff}}$ onwards, the spectrum is $P(v)$. This energy corresponds to $r_c = R$, and sets the position of the low energy break.

If the magnetic clouds have different shapes, there may be a smoother break. For example, it is easy to show that spherical cloudlets offer an effective volume

$$V_{\text{eff}}(r_c) = V_0[3(r_c/R) - 3(r_c/R)^2 + (r_c/R)^3] \quad (2)$$

Again, at low energies, the term r_c/R dominates.

Summing up, this mechanism flattens the low energy spectrum by a half-power law; the specific shape of the break depends on the geometry of the magnetic inhomogeneities, and on the dispersion of their sizes. The fact that the radiated power per electron must in this case be multiplied by a factor γ has been noticed by Tucker¹, but not exploited, as far as we know. From the low energy break onwards, the spectrum follows its theoretical $P(\nu)$. A second break then ensues when the energy losses of electrons by radiation become important. It is well known that this happens for a Lorentz factor $\gamma = 5.2 \times 10^8 H^{-2} T_{\text{eff}}^{-1}$, in Gaussian units. In this case, of course, T_{eff} is not equal to T , the lifetime of the nebula, but is the fraction of T which the electrons spend in the magnetic clouds. For high energy electrons, and in the case of spherical cloudlets, $T_{\text{eff}} \sim 2TR/\Lambda$. In this expression Λ is defined in such a way that $\Lambda - 2R$ is the customary mean free path. When $2R = \Lambda$, the field is uniform, and setting $2R > \Lambda$, of course, does not make any sense.

As an application of what we said above, we will show that it is easy to find a reasonable set of parameters H , R , Λ which can reproduce the gross features of the spectrum of the Crab Nebula. For gross features we mean first, the radio spectrum, which extends up to about $\nu = 3 \times 10^{13}$ Hz, with power law index not too different from 0.3; second, the optical spectrum, with a power law index compatible with the value 0.8; third, the high energy spectrum, from 3.2×10^{14} Hz on, where a power law index of 1.3 can well represent the data².

We then have the following equations

$$\begin{aligned} \nu_1 &= 1.2 \times 10^6 H_1 \gamma_1^2 = 3 \times 10^{13} \\ \nu_2 &= 1.2 \times 10^6 H_1 \gamma_2^2 = 3.2 \times 10^{14} \\ \gamma_1 &= 7 \times 10^{-4} H_1 R \\ \gamma_2 &= 0.009 (\Lambda/R) H_1^{-2} \end{aligned} \quad (3)$$

As usual, H_1 indicates the component of the magnetic field normal to the electron trajectory. We have assumed that the magnetic fields are randomly oriented, and therefore $H_1 = \sqrt{(2/3)}H$. Setting the magnetic field $H = h_0 \times 10^{-3}$ gauss, gives the following:

$$\begin{aligned} R &= 2.44 \times 10^{11} h_0^{-3/2} \text{ cm} \\ \Lambda &= 1.03 \times 10^{13} \text{ cm} \\ \Lambda/R &= 42.4 h_0^{3/2} \end{aligned} \quad (4)$$

From the last expression we see that $h_0 \geq (21.2)^{-2/3} = 0.13$ for the model to make sense.

To verify that the energetic requirements of the model are tolerable we estimated E_e , the total energy of the electrons, E_h , the total energy of the magnetic field, and $\delta = E_h/E_e$, which should tell how far we are from equipartition. We assumed, for simplicity, a uniform distribution of spheroidal cloudlets. In this case, the total volume of the clouds is given by $V_0(R/\Lambda)$, since the average distance between clouds is approximately given by $(R^2\Lambda)^{1/3}$. The distance we assumed is 2 kpc, and V_0 , the total volume of the (radio) nebula, is $3 \times 10^{55} \text{ cm}^3$, according to the data given by Apparao³. With these assumptions we get: $E_e \sim 2.6 \times 10^{50} \text{ erg}$; $E_h \sim 2.8 \times 10^{46} h_0^{0.5} \text{ erg}$ and $\delta \sim 1.1 \times 10^{-4} h_0^{0.5}$. For different shapes and dispositions of the clouds (filaments or sheets) these parameters would depend on different powers of h_0 , and we feel that the situation could well move closer to equipartition.

The foregoing model is not as limited in scope as might appear at first sight. We will now show that a system of filaments with a more general magnetic field distribution, must give, to a first approximation, a similar flattening of the spectrum. Consider the radiation from a single filament, where the magnetic field as a function of the axial distance r is given by the law $H \propto r^{-\alpha}$. There are two restrictions: α must ensure a sufficiently fast decrease of the magnetic field, and because an infinite magnetic field is nowhere acceptable, we must expect that the field is truncated at $r = R_0$. The character of the spectrum is most easily seen if we suppose that $H = 0$ for $r < R_0$. We then make rather crude approximations, with the result that the total power radiated is given by the law

$$L(\nu) \propto \int_{r_{\min}}^{\infty} \gamma^2 H_1^2 \gamma_v^{-m} \gamma_v \nu^{-1} r dr \quad (5)$$

Here, $r_{\min} = \max(R_1, R_0)$, where $R_1 \propto \nu^{1/(2-3\alpha)}$ and m is the index of the electron distribution, which we take as 2.6. The expression for R_1 , the counterpart of the geomagnetic cutoff, is easily found by setting the cyclotron radius equal to the axial distance r , and keeping in mind that

$$\nu \propto H_1 \gamma^2 \propto H_1 (Hr)^2 \propto r^{-\alpha} (r^{1-\alpha})^2$$

It then seems that for $R_1 < R_0$, or $\nu > aR_0^{(2-3\alpha)}$, where a is a suitable constant, the spectrum follows the customary power law $\nu^{-(m-1)/2}$. On the contrary, for $\nu < aR_0^{(2-3\alpha)}$, there appears an extra factor ν^{β} , where

$$\beta = \frac{2 - \alpha(m+1)/2}{(2-3\alpha)}$$

The numerical value of β depends both on α and on m . For $m = 2.6$, it is easy to see that β has the asymptotic value of 0.6, and for $\alpha = 2$ it takes the value 0.4.

We have here merely tried to show that it is possible to achieve a reasonable fit for the spectrum of the Crab Nebula, without worrying, for the present, about other features, and without attaching too much weight to the values obtained for R and Λ . We thus lose equipartition, but we find a simple explanation for two breaks, and, perhaps more important, we show that an injection spectrum of electrons with power law index 2.6 is acceptable.

It is interesting to note that we are left with one free parameter, namely h_0 . With this parameter in hand, we can adjust the magnetic field so that it is not in conflict with the γ -ray observations of the Crab Nebula⁴. We have numerically estimated the Compton-synchrotron radiation by means of the complete formulae found in the review paper by Blumenthal and Gould⁵. A value of $H \sim 10^{-2}$ gauss is largely sufficient to avoid an excess of high energy γ rays, whereas $H = 10^{-3}$ gauss is on the border line.

Whether the foregoing model is really valid for the Crab or not, we think that the situation described here may occur in many synchrotron emitting sources.

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Thorium in the spectrum of HR465

THE peculiar A star HR465 is a spectrum variable¹. Considerable attention^{2,3} has been given to the spectra of this star obtained by W. P. Bidelman during 1960–61, when the lines of the rare earth elements attained their maximum intensity; Bidelman has kindly placed this material at our disposal. Among the remarkable aspects of the HR465 spectrum is the fact that it is the only star for which a firm statistical basis exists for the identification of uranium⁴.

Bidelman (unpublished) has assigned a moderate spectral feature in HR465 at $\lambda 4019.12$ to Th II $\lambda 4019.13$, which is the strongest accessible thorium line. The occurrence of this line in stellar spectra is very unusual. We base this statement on an extensive series of wavelength measurements made by C.R.C. and G.C.L.A. on $2.4 \text{ \AA mm}^{-1}$ plates taken at the Dominion Astrophysical Observatory. Combining these results with previous work^{2,5}, we have measured wavelengths in some 50 Ap and Am spectra, many of them very rich and sharp-lined, and only HR465 shows a line within $0.04 \text{ \AA}$ of $\lambda 4019.13$.

The thorium identification can be based on more than the single line $\lambda 4019$. We have re-examined the statistics of wavelength coincidences using the Th II wavelengths and intensities given by Meggers, Corliss and Scribner⁶. Coincidences within ± 0.04 of stellar wavelength with Th II are significant at the 95% confidence level or higher. The highest significance (99.5% confidence) was based on four coincidences within $\pm 0.02 \text{ \AA}$ using a list of seven Th II lines with laboratory intensities greater than 1,000. These results are somewhat more favourable than those obtained earlier².

We have obtained an abundance for thorium in HR465 at rare-earth maximum from this line using the method of Cowley and Hartoog⁴. The gf values were taken from Corliss and Bozman⁷ or computed from the data in Meggers *et al.*⁶ using atomic data supplied by Corliss.

This method shows a remarkable consistency. For example, we determined the thorium–neodymium abundance ratio using $\lambda 4018.69$ of Nd II, and the thorium–gadolinium ratio using $\lambda 4013.80$ of Gd II. When these ratios were converted to absolute abundances using Aller's¹ analysis of HR465, the results are within 0.2 in the logarithm. Other abundance determinations⁸ show a similar self consistency.

The absolute abundance that we derive for Th is 4.4 (log $H = 12.0$). This should be compared with the uranium abundance of 4.6, which we now revise from ref. 4 on the basis of a new second ionisation energy for uranium⁹. We believe this result to be correct to slightly better than an order of magnitude.

If we assume that the present Th–U ratio of -0.2 dex ($\pm$ roughly 0.7 dex) in HR465 is the result of radioactive decay following the nucleosynthetic event, then we can say that the event must have taken place more recently than the time of the birth of the Galaxy, some 10^{10} or more years ago.

Certain non-nuclear^{10,11} theories of the origin of abundance anomalies in Ap stars would obfuscate any conclusion of this kind. But these theories have not been developed to the point where they can make definite prediction of the relative abundances of heavy nuclides. On the other hand, it has been shown⁸ that the relative abundance pattern of the heavy nuclides in HR465 resembles the prediction of the r-process. We shall, therefore, proceed with a nuclear interpretation, which at present provides the most complete and detailed basis for an understanding of the observations.

Cameron¹² gives 0.67 for the logarithm of the solar system thorium–uranium ratio. Our result differs from this by 0.87 in the logarithm. If these figures could be taken at their face value, there would be a clear indication from the work of Blake and Schramm¹³ that the HR465 material was processed less than 10^9 yr ago while the Solar System r-process ceased more than 10^9 yr ago. Unfortunately, the uncertainty of the

present determination will not permit this interesting conclusion, and we must content ourselves with the statement that thorium and uranium were made in HR465 at some time more recently than the formation of the oldest globular clusters.

The significance of this crude cosmochronological result lies in the fact that it depends on observations of an object outside of the Solar System.

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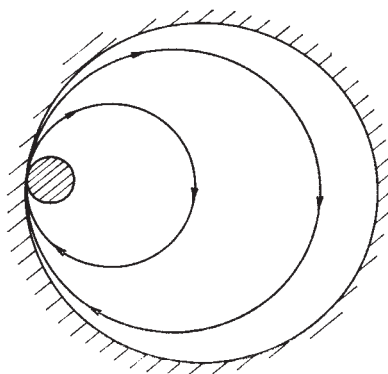
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Controlled artificial generation of geomagnetic pulsations

THERE has been a shift away from purely passive studies of the ionosphere and magnetosphere to more active studies in which the response of these regions to a controlled man-made disturbance is determined by experiment^{1,2}. One area of research where active experiments may be particularly useful is in the study of ultra low frequency (u.l.f.) waves in the ionosphere and magnetosphere. We do not yet completely understand these waves from observations of the geomagnetic pulsations they produce because of the great difficulty in separating source and propagation effects. This problem is particularly acute for the u.l.f. waves generating Pc 1 geomagnetic pulsations (0.2–5 Hz) where the wavelengths are small compared with the dimensions of the magnetosphere. What is needed is a controlled

Fig. 1 Large scale representation of the island/circular sea model. The island is connected to the shore of the sea by a narrow insulating neck of length 100 m at the point of closest approach. Other relevant dimensions are: island radius 10 km, sea radius 100 km, sea depth 50 m, total current between electrodes on either side of the neck 3,000 A. Two of the circular current flow lines in the sea are indicated by arrows.



source of u.l.f. waves which would enable source and propagation effects to be separated. We believe we have found a technically feasible and comparatively inexpensive method for the controlled artificial generation of u.l.f. waves.

A number of suggestions have been made for such a generator, suggestions include a large ground-based current loop, or a large horizontal antenna³⁻⁷. Artificial stimulation of u.l.f. waves by direct modification of currents in the lower ionosphere has been proposed⁸. Another method uses very low frequency (v.l.f.) radio transmissions into the magnetosphere to stimulate u.l.f. waves⁹⁻¹¹. The ground-based current loop method would probably be one of the most reliable and versatile but, to generate observable pulsations, the area of the loop must be large and the

between electrodes which are, for simplicity, assumed to completely cover either side of the neck. The current distribution for a given potential difference is obtained by solving Laplace's equation using standard conformal mapping techniques. The flow lines are circular and the sea can be divided into circular current elements of infinitesimal width. The ionospheric magnetic field is obtained by integrating over the contributions of all elements. The sea is required to be bounded so that the inductance is finite. The inductance calculation is difficult, and an estimate was made from measurements on a scale model.

The relevant parameters are as follows: resistance 0.246Ω , maximum current density 0.6 A m^{-2} , power at 1 Hz – 2.39 MW , self inductance $\approx 20 \text{ mH}$. Magnetic fields generated in the ionospheric E region are shown in Fig. 2. At u.l.f. frequencies, simple lumped circuit analysis may be applied; at 1 Hz , the circuit has an impedance of 0.27Ω and the power required to pass a current of $3,000 \text{ A}$ through the seawater is 2.39 MW . The maximum current density (at the electrodes) is 0.6 A m^{-2} ; over most of the sea the current density is much smaller: on the far side of the island it is only 0.01 A m^{-2} . The amplitude of the magnetic field generated is more than 2.5γ over $10,000 \text{ km}^2$ at an altitude of 100 km . This magnetic field amplitude is greater than that of most naturally occurring micropulsations.

There are several idealisations in our model. Perhaps the most important is the assumption that the sea boundaries are insulating. Clearly, the currents through the neck and adjacent sea floor must be minimised. Seawater conductivity is typically 4 S m^{-1} and for a location where the island, neck, and sea floor consists primarily of solid rock such as granite, with a conductivity of less than 10^{-2} S m^{-1} , approximately 50% of the power is lost by conduction through the neck. This could be reduced by insulation placed behind the electrodes. Alternatively, a freshwater lens beneath the neck and island would reduce the conductivity substantially. Furthermore, currents induced in the sea floor by the changing magnetic field generate an opposing magnetic field. For a conductivity of 10^{-2} S m^{-1} , and frequency 1 Hz , the skin depth is 5.0 km , and for a current loop the maximum axial magnetic field produced at an altitude of 100 km will be reduced by a factor of 6 from the ideal value³ obtained assuming zero conductivity. Even reduced by this factor the fields generated are still greater than most naturally occurring micropulsation amplitudes.

The other idealisations in the model are less likely to be important in practice. The shapes of the peninsula and sea are unimportant, as shown in model calculations, provided the area enclosed by the current flow lines is large. There exist a number of saline lakes and enclosed seas that would be suitable; for example, the Great Salt Lake in the USA. The use of electrodes which completely cover the neck is usually impractical. Smaller electrodes will produce an increased voltage gradient in the immediate vicinity of the electrodes, possibly necessitating fencing to exclude sea life. Farther from the electrodes deviations from the idealised case will be insignificant. We emphasise that the resulting voltage gradients are very small. For example, in our model a swimmer opposite the neck of the island would experience a voltage difference of 0.03 V from head to toe (a distance assumed to be 2 m).

An important distinction between this new version of the ground-based current loop method for generating u.l.f. waves and the original method is its use of the conducting properties of the 'ground' (that is, seawater), resulting in reduced construction and operation costs and more available locations. Once a suitable peninsula has been located, the insertion of electrodes, switching circuitry and transformers and the provision of a modest amount of power ($\sim 1 \text{ MW}$) should allow the generation of distant u.l.f. waves of observable magnitude.

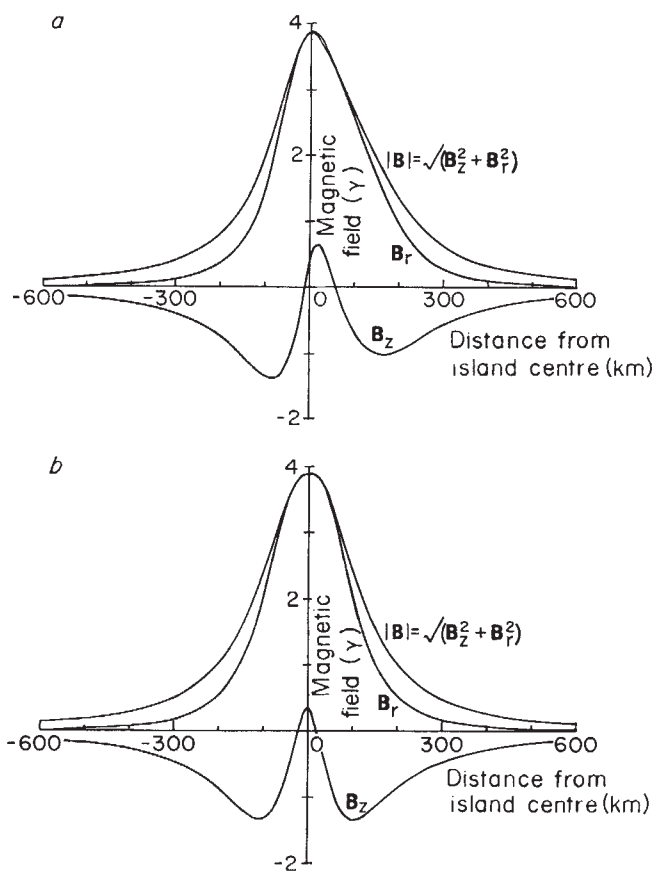


Fig. 2 The magnetic field generated at 100 km altitude. *a*, Distance from island centre measured perpendicular to the shore; *b*, distance from island centre measured parallel to the shore.

power requirement is many MW. We here propose a new version of the ground-based current loop method, in which an electric current is passed around a peninsula in an enclosed sea or large saline lake. We describe model calculations which demonstrate that significant magnetic field variations can be generated in the lower ionosphere for powers of the order of 1 MW . It has been shown theoretically that this can produce u.l.f. waves in the ionosphere^{3,7}.

The model analysed consists of a small circular island connected to the shore of a large circular sea by a narrow neck (Fig. 1). All sea boundaries are assumed to be insulating. Physical dimensions are chosen as follows: island radius 10 km , sea radius 100 km , neck length 100 m , sea depth 50 m . An electric current flows around the island

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Reality and nature of a Sun-weather correlation

WILCOX *et al.*^{1,2} have claimed that some influence associated with the solar magnetic sector structure produces an effect in the terrestrial troposphere, which is manifested by changes in the vorticity area index (VAI). (The VAI is a measure of the strength of the cyclonic circulation in the Northern Hemisphere.) The phenomenon was revealed by means of a superposed-epoch analysis of the VAI data which made use of 54 key days, determined by the solar magnetic data, and produced a mean variation about the key days (Fig. 1—our reconstruction of Fig. 5 of ref. 2). Wilcox *et al.* concluded that the effect lasts for a few days and introduces changes of up to 10% in the VAI, but occurs only in the winter months.

Reports of short term Sun-weather correlations have been greeted with scepticism by many. We have, however, subjected the VAI data (provided by W. O. Roberts; determined for the 3×10^4 Pa (300 mbar) level) of the original analysis to a variety of statistical tests, and we have requested and obtained a subdivision of new data that have been reported³ in furtherance of the claim. (J. M. Wilcox and L. Svalgaard, personal communication. They extracted from the analysis in ref. 4 results both for the 81 new cases included there, and for the 46 whose key days could be clearly identified from spacecraft observations alone.) We find ourselves obliged, however, to accept the validity of the claim by Wilcox *et al.*, and to seek a physical explanation.

In the course of our statistical tests, we had determined that the alleged solar 'signal' was stronger during periods of relatively strong meteorological 'noise' (as measured by the amplitude of VAI variations, indicative of transient large scale weather systems); a 'signal' amounting to a 30% change of VAI was detected in the case of a superposed-epoch analysis of the 12 'noisiest' key days, for example. This had seemed to indicate that the alleged signal might be merely residual noise, inadequately cancelled in the superpositioning. If the reality of the correlation is accepted, however, quite a different inference must be drawn. Either the meteorological noise is, in large part at least, the solar signal, or else the solar influence (whatever its nature may be) must somehow modulate the meteorological noise to produce its signal. The first option is generally discredited

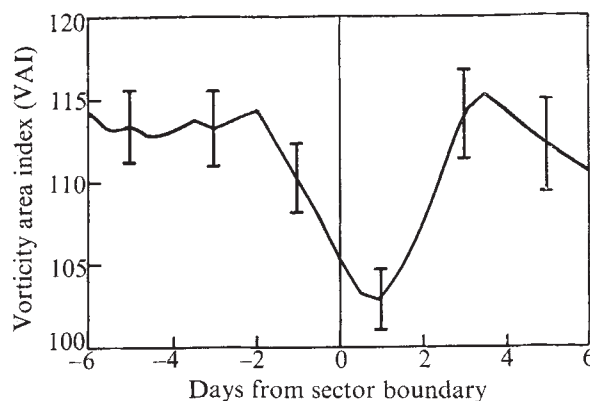


Fig. 1 Variation of vorticity area index (VAI, in units of 10^6 km², determined for the 3×10^4 Pa pressure level) with days measured from the passage of a solar magnetic sector boundary past the Earth: a superposed-epoch result incorporating VAI data associated with 54 sector boundaries, reconstructing the variation depicted by Wilcox *et al.*²

on grounds of simple energetics, and it is probably ruled out by observational evidence, even if 'trigger' mechanisms are invoked. The second option does not suffer from these difficulties, however, and we investigate it here.

Phase modulation is the simplest kind we can propose and we have tested it by simulation methods. We suggest that the solar influence can selectively accelerate and/or decelerate the natural amplitude variations of VAI in such a way that minimum VAI tends to be found near to the times of the passage of solar magnetic sector boundaries past the Earth, rather than being randomly distributed. The physical mechanism by which such phase shifts might be introduced remains unknown.

To test this hypothesis by simulation, we have chosen at random 54 artificial key dates (and other numbers of artificial key days) in a variety of ways from among all the winter days in the period January 1, 1964 to December 31, 1970—the period used by Wilcox *et al.*² We then shifted the key days forwards or backwards by 12 or 24 h, to minimise the VAI that would have been obtained on the key day. Both the purely random and the adjusted sets of artificial key days were then used in superposed-epoch analyses of VAI. The phase adjustment was found to produce patterns having a well defined signal from the originally random data (Fig. 2). The upper set of traces depicts results for 54 key days; the lower set depicts results for 131 key days, the number now in use³. Between the two sets, on the right, is the original signal.

The simulation signal we have produced in this way constitutes a signature determined by, and characteristic of, the VAI data, in combination with the rules of phase adjustment alone. The solar influence, whatever its nature, produces a signature of its own that is clearly of much the same character. The signatures could be forgeries of one another; but, if they are not, the solar influence must be producing its signature very largely by a phase shifting of meteorological noise.

We are at present testing variants on the rules of simulated phase shifting in order to duplicate more successfully the solar-induced signature and to determine the range of rules that might apply. Hypotheses alternative to the phase-shift hypothesis, but consistent with our basic contention that meteorological noise is being modulated, are also under consideration. Our detailed results will be reported shortly.

The concept of a phase-shifting or similar modulating mechanism very largely avoids, *a priori*, difficulties normally associated with the energetics of a Sun-weather relationship: the usual sources and sinks of meteorological energy continue to be called on, and it is only the timing of the

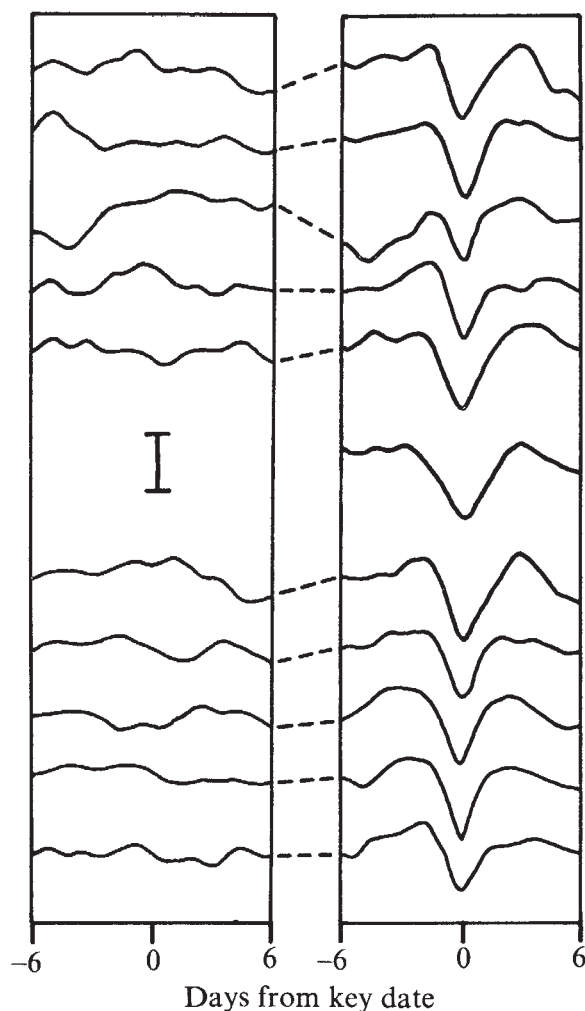


Fig. 2 Variation of VAI with days measured from key dates that are selected at random (left panel) and from key dates that are adjusted as described in the text from the randomly selected key dates (right panel). The upper five curves use 54 key dates and the lower five curves use 131 key dates. The vertical bar in the left panel represents 10 units (of 10^5 km^2) in the VAI. The curve opposite it in the right panel is the curve of Fig. 1, replotted on the present scale and shifted one day to the left (to match the criteria imposed for the adjustment of the randomly selected days).

calls or the detailed development of their consequences that need be altered. To the extent that the naturally occurring winter noise in VAI may occur because of meteorological instability, and to the extent that the meteorological instability may itself be sensitive to the processes that produce the presumed modulation, those processes may occasionally raise an incipient instability above, or suppress it below, the threshold level for rapid growth. The solar influence might then have a marked effect on the form (and not just the timing) of the weather, but we cannot conclude anything on the basis of the available data. The importance of the particular Sun-weather correlation discussed here may then lie not so much in the prediction of abnormal solar effects as in the improved prediction of normal meteorological processes.

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Conductivity and rectification in metal halide intervalence mixtures

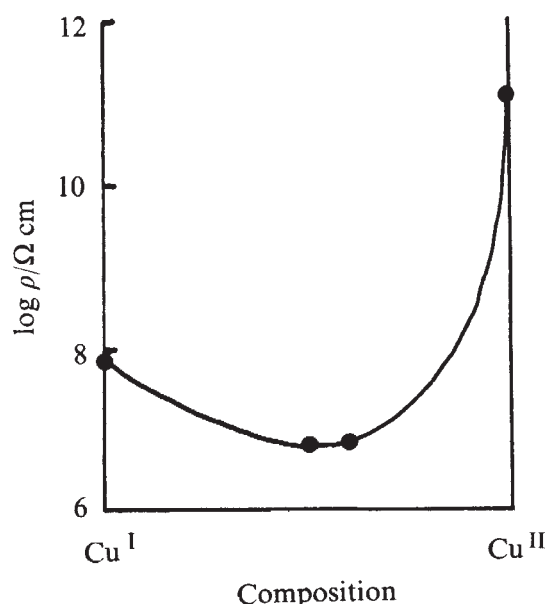
INTERVALENCE complexes contain two or more atoms of an element in different oxidation states; they have been collated and characterised as a group having properties peculiar to their valence structures¹. Thus, the conductivity of a mixed-valence salt often exceeds that of either single-valence progenitor. We present here novel observations of just such effects in pressed-disk mixtures of copper(I) and copper(II) chlorides. The test is stringent because the (I) and (II) states have different geometries, tetrahedral and octahedral, and fall on the borderline of class 1-class 2 behaviour¹. The disfavoured geometries from a Cu(I)-Cu(II) electron transfer event can, however, probably be mitigated as follows: disks pressed from ground powders can be used without thermal treatment, doubtless leaving single-state domains with dimensions close to those of the original particle sizes, and even though true intervalence can occur only at their boundaries, mixed and intermediate geometries are also likely. Figure 1 shows a tenfold enhancement of the Cu(I) value and ten thousand-fold enhancement of the Cu(II) value.

Since the conductivities are all low, in CuCl and the mixtures, electron hopping is the indicated mechanism, though anion migration has been proposed to explain the CuCl₂ process².

The two-centre electron-transfer process³ has provided a basis for the interpretation and prediction of solid-state properties in the systems discussed here. We have assumed that chloride bridges between the various metal ions assist in lowering the barrier to hopping. We also aimed to adjust the effective Fermi levels in different regions by the introduction of oxidising or reducing chlorides—FeCl₃ and SnCl₂, respectively—themselves linked by chloride bridges to the intervalence host-matrix conducting system. Thus an FeCl₃-doped disk pressed to an SnCl₂-doped disk should generate excesses of the appropriate Cu valencies, which then annihilate at the contact leaving an effectively p-n junction; this proved to be the case.

Equimolar Analar white CuCl and brown CuCl₂ (dehydrated dihydrate) were ground together in a glovebox

Fig. 1 Log of resistivity/ $\Omega \text{ cm}$ against composition for CuCl-CuCl₂ disks.



containing dry nitrogen. An ordinary pellet press for infrared spectroscopy samples was used to obtain mottled, olive-green disks flecked with white and brown. A mixture containing 1% SnCl_2 was tamped down using a momentarily applied half-ton pressure, then a mixture containing 1% FeCl_3 was added as a further discrete layer, and a pressure of 12 ton was applied for several minutes. Disks 1.5 cm in diameter were released to a dry nitrogen atmosphere and $\sim 0.2 \text{ cm}^2$ platinum contacts were silver-pasted on. For handling they were either sealed in wax or suspended in a desiccator with leads, and caged for measurements with shielded leads to an electrometer. Disk thicknesses were 1–3 mm.

With 1% doping, relative currents of 2.0 (FeCl_3 negative) and 1.6 (SnCl_2 negative) were observed. With 5% doping the relative values were 5.7 and 1.8, respectively. To establish that Sn^{4+} and Fe^{3+} space charges gave rise to the junction, we conjoined disks with 55:45 Cu(I)–(II) compositions. Nil rectification ensued.

Neither the actual conductivities nor the rectifications are themselves particularly impressive, but they illustrate far-reaching principles, from bottles of four reagents which just happened to be at hand. We have in mind applications to dithiolates⁴ which, first, conduct relatively well as single components⁵, second, exist in a wide variety of oxidation states⁴ useful for intervalence mixing, third, show a wide range of reducing and oxidising ('donor' and 'acceptor') powers⁴ useful for doping, and, in some solid state charge transfer adducts (D.R.R. *et al.*, ref. 7), show clear photovoltaism. The combination of these factors predicate good prospects for enhancing the efficiencies of photoelectric energy conversion. Finally, pressed disks (already established as satisfactory conducting systems^{5,6}) must be cheaper than the fused systems used in technology.

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Precise determination of critical features in radiohalo-type coloration of biotite

HIGH precision techniques of photomicrography and densitometry are here used to investigate the coloration of artificially α -irradiated biotite. A computer simulated spherical radiation pattern is found to produce coloration peaks, contrary to the negative results of earlier investigators. A clearer understanding of the relationship between the usually defined α ranges and 'coloration ranges' is obtained as well as the elucidation of several other features of radiohaloes. In spite of more than a half a century of investigation, the parameters of α -induced radiation damage in biotite are still only crudely known. The principal parameter, the 'range' of the α particle, has been studied primarily by means of the uranium radiohalo and its close relatives (ref. 1; see also, refs 2 and 5). The coloration rings of these haloes lend themselves to direct, though somewhat inexact, measurements of the ranges of α particles of various energies. Early investigators also made use of measured α ranges in air and then converted these to ranges in biotite. Recently, Gentry^{2,3} has made a substantial number of direct 'range' measurements in biotite and other minerals by bombardment with accelerator-produced α particles.

In spite of all previous work, a number of perplexing and basic concepts remain to be elucidated. We now report a series of precision measurements on biotite, from the Faraday pegmatite at Bancroft, Ontario, Canada⁴, which was irradiated by α particles from the 5-MeV Van de Graaff accelerator at Wayne State University. Our investigations have explained a number of new features not previously recognised or, in some cases, recognised but not understood. We have been able to prove by direct irradiation techniques that a spherical, as distinct from a linear, radiation pattern will produce a series of coloration rings in biotite. Early experimenters¹ arrived at a puzzling negative result when they attempted by indirect means to prove this. We have also obtained the precise relationship between ' α range' as determined by the biotite coloration pattern and the true range. Moreover, we have made a high precision measurement of the range of the two most energetic uranium α particles, the 7.69-MeV and 6.00-MeV decays. A number of other puzzles have also been solved.

Blocks of biotite were polished to have a smooth face of $\sim 1 \text{ cm}$ by 2 cm perpendicular to the cleavage plane. The

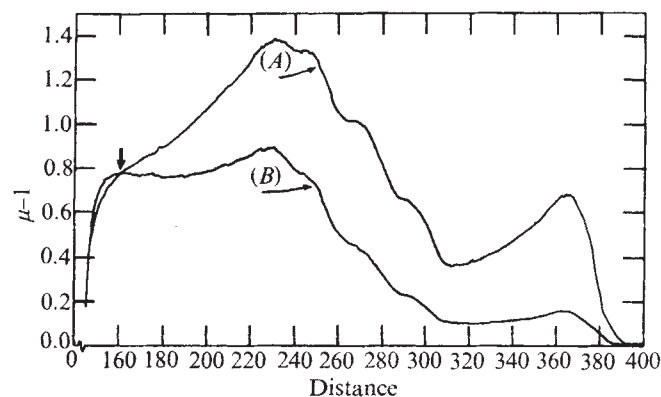


Fig. 1 A, The dimensionless optical absorption coefficient for α -irradiated biotite. B, The spherical absorption coefficient as derived from equation (3), arbitrarily normalised to A at the arrow. Notice the slight leftward shift of the curve B peaks due to the r^{-2} factor. The units along the ordinate are dimensionless since the absorption coefficient, μ , is expressed in units of the absorption coefficient for the natural (unirradiated) mica. The numbers along the abscissa are arbitrary, zero not being the mica edge but the start of the densitometer trace of the negative. The size of one abscissa unit is $0.147 \mu\text{m}$. The first peak (4.2 MeV) occurs at ~ 230 on the abscissa.

usual techniques of lens polishing worked well, but it was necessary to clamp the block tightly to keep the working face from fraying. The irregularity of the finished face was $\leq 1 \mu\text{m}$.

While still clamped, the finished face was irradiated by an α beam striking it normally. The size of the beam spot was $\sim 7 \text{ mm}^2$ and the intensity varied from 2×10^{-9} to 10^{-8} A as required. The Wayne State University accelerator produces highly monoenergetic particles, so the energy spread of several keV in the beam was insignificant for our purposes.

After irradiation the biotite was cleaved into flakes approximately $20 \mu\text{m}$ thick. For visual and photographic inspection of the thin sections, a Leitz Ortholux was used at a fixed magnification of 340.5, which we did not vary. After several unsuccessful photomicrographic results, we found that Kodak Contrast Process Pan 4155 film produced the best resolution with high contrast. In order to compensate for the nonlinear properties of this film (any film is, of course, nonlinear in some tone ranges), we calibrated it against a known photographic density wedge. All the results we report have been adjusted for the nonlinearity of the film.

Our photomicrographs were scanned by an Optronics Photoscan System P-1000 densitometer whose output was fed

directly to magnetic tape. The tape was then run in conjunction with a program which reduces and analyses the raw data in several ways. We used the WSU IBM360/67 for all our work.

The key to understanding the densitometer tracings is to choose the appropriate physical variable for the computer output. This variable is the optical absorption coefficient of biotite, which is independent of the thickness of the specimen section. We express all our absorption coefficient results in units of the absorption coefficient for natural biotite, μ_N . If we designate the total optical absorption coefficient as μ_{tot} , we define our coefficient as $\mu \equiv \mu_{tot}/\mu_N$. Hence $\mu = 1$ is the value for the natural mica and α irradiation produces values for which $\mu > 1$.

In Fig. 1, curve A shows the plot of the absorption coefficient, μ , against distance from the edge of the mica section. We see five easily discernible peaks. (Actually there are 8 α -decay parent nuclei in the ^{238}U chain, with 11 significant α decays. Because the multiple α branches of the multi-branched nuclei are very close in energy, however, the literature has traditionally referred only to 8 α energies, of which only 5 can generally be resolved.) It is, however, critical to keep in mind that this is a plot of μ for a linear superposition of the eight energies. Haloes involve spherical superposition, which means that the

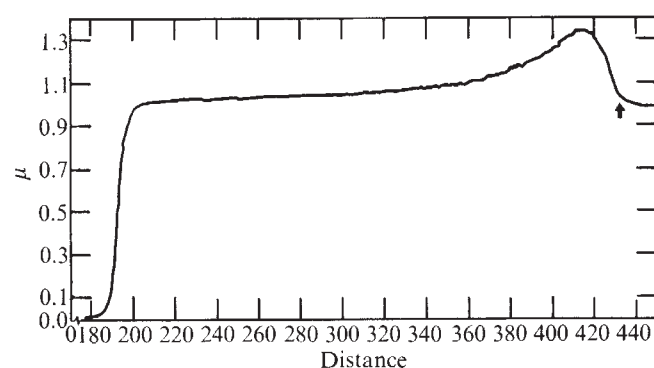


Fig. 2 The optical absorption coefficient curve for an α energy of 7.69 MeV. See Fig. 1 caption for an explanation of abscissa and ordinate values. The arrow points to the extrapolated ionisation range.

radiation intensity drops by r^{-2} as the distance from the halo centre increases. The early experimenters¹, realising that halo darkening depended in some way on α -particle ionisation, attempted to use ionisation curves in air to produce a spherical halo pattern. By taking a linear superposition of eight ionisation curves, and then multiplying them by r^{-2} , they attempted to reproduce a halo. They had negative results. We now understand the reasons for their failure. They failed partly because: they used poor ionisation curves to start with; they falsely assumed the air-biotite conversion was independent of energy⁵; and they did not understand how true range related to 'coloration range'. Principally, however, they failed because ionisation is not the correct variable to display if one wishes to investigate coloration peaks. The correct variable, as stated above, is the absorption coefficient.

According to the elementary theory of F centres⁶, the absorption coefficient will increase with radiation according to

$$\mu = \mu_{max}(1 - \exp(-kd)) \quad (1)$$

where μ_{max} is some characteristic maximum value, k is characteristic of the substance, and d is the particle dose. Deutsch *et al.*⁷ experimentally verified in 1957 that such a curve holds very well for the α darkening of a wide variety of biotites. Of course, ionisation at any fixed point along the α path is proportional to the intensity of the primary radiation. From curve

A in Fig. 1 we determine the dose which is

$$d = -\frac{1}{k} \ln\left(1 - \frac{\mu}{\mu_{max}}\right) \quad (2)$$

where μ_{max} has been determined for our biotite to be 2.87 ± 0.02 by saturation dosing. Thus we see from equation (2) that the relationship between ionisation and darkening is simple only when the dose is small, and thus proportional to μ itself. To convert correctly the linear μ curve (A) in Fig. 1 into a simulated spherical optical absorption curve (B) we must multiply the dose by a factor r^{-2} , or, with r_0 an arbitrary normalisation point,

$$\mu_{sph} = \mu_{max} \left\{ 1 - \exp\left[\left(\frac{r_0}{r}\right)^2 \ln\left(1 - \frac{\mu}{\mu_{max}}\right)\right] \right\}$$

Our calculation took into account the fact that the above equation is not correct if artificial irradiation contributes darkening ionisation to an already naturally dark mica. The correct formula, used in our computations, is

$$\mu_{sph} = \mu_{max} \left\{ 1 - \left(1 - \frac{1}{\mu_{max}}\right) \exp\left[\left(\frac{r_0}{r}\right)^2 \ln\left(\frac{\mu_{max} - \mu}{\mu_{max} - 1}\right)\right] \right\} \quad (3)$$

We note that the peaks still exist in curve B, consistent with what is actually observed, and contrary to the results of previous investigators. Also, it is now obvious why Gentry found the 'ranges' in haloes somewhat shorter than those in artificially irradiated biotite. Equation (3) causes precisely this effect.

Figure 2 shows the μ curve for the single α energy of 7.69 MeV, at a low dose. Note the great similarity in shape to a typical ionisation curve^{8,9}. This is because we have, as a first approximation to equation (1)

$$\mu \propto d \propto \text{ionisation} \quad (4)$$

We can also see relationship of the peak or 'coloration range', to the usually defined ranges of the α particle. The extrapolated ionisation range, R_i (ref 8 and 9) is shown in Fig. 2. This range is very similar to the mean range, $\bar{R}$, but differs slightly from it.

Note that R_i is not the same as the 'coloration range', R_c , which actually corresponds to the peak of the ionisation curve. Nor is R_i the same as the frequently used 'threshold' or 'coloration extinction' range determined by the visual determination of the limit $\mu \rightarrow 1$, this latter range being dose dependent^{2,3} whereas R_c and R_i are not. (Halo investigators from Joly on have, of course, been aware that the ionisation ranges of α particles in air gave, on conversion, mica ranges corresponding roughly to the outer foot of the coloration peak.) Since, in practice, coloration extinction is observed near, but above, the value $\mu = 1$, a 'threshold' range can be less or greater than an ionisation range depending on the dose. (To be absolutely precise R_i should be computed from the ionisation curve, whereas we used the μ curve of Fig. 2. When, however, $\mu \sim 1$, equation (4) shows that the choice of curves is immaterial.)

We have found the ranges to be

$$R_c(7.69 \text{ MeV}) = 32.2 \pm 0.2 \mu\text{m}; R_c(6.00 \text{ MeV}) = 21.4 \pm 0.2 \mu\text{m} \\ R_i(7.69 \text{ MeV}) = 35.1 \pm 0.2 \mu\text{m}; R_i(6.00 \text{ MeV}) = 23.7 \pm 0.2 \mu\text{m}$$

which we believe to be the most precise determinations of R_c and R_i yet given, as well as the first time a clear understanding of the different ranges has been achieved.

The precisely defined and carefully determined ranges given above are a necessary first step towards our further goal of comparing ancient α ranges with recent values in order to test the long term constancy of nature^{4,5,10}.

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'1/f noise' in music and speech

THE power spectrum $S(f)$, of many fluctuating physical variables, $V(t)$, is '1/f-like', varying as $f^{-\gamma}$ ($0.5 \lesssim \gamma \lesssim 1.5$), over many decades of frequency (see ref. 1 for review). We have found that loudness fluctuations in music and speech, and pitch (melody) fluctuations in music exhibit 1/f power spectra.

$S(f)$ is related to the autocorrelation function $\langle V(t)V(t+\tau) \rangle$ by the Wiener-Khinchine relationships². If $V(t)$ can be characterised by a single correlation time τ_c , $V(t)$ is correlated with $V(t+\tau)$ for $|\tau| < \tau_c$, and is independent of $V(t+\tau)$ for

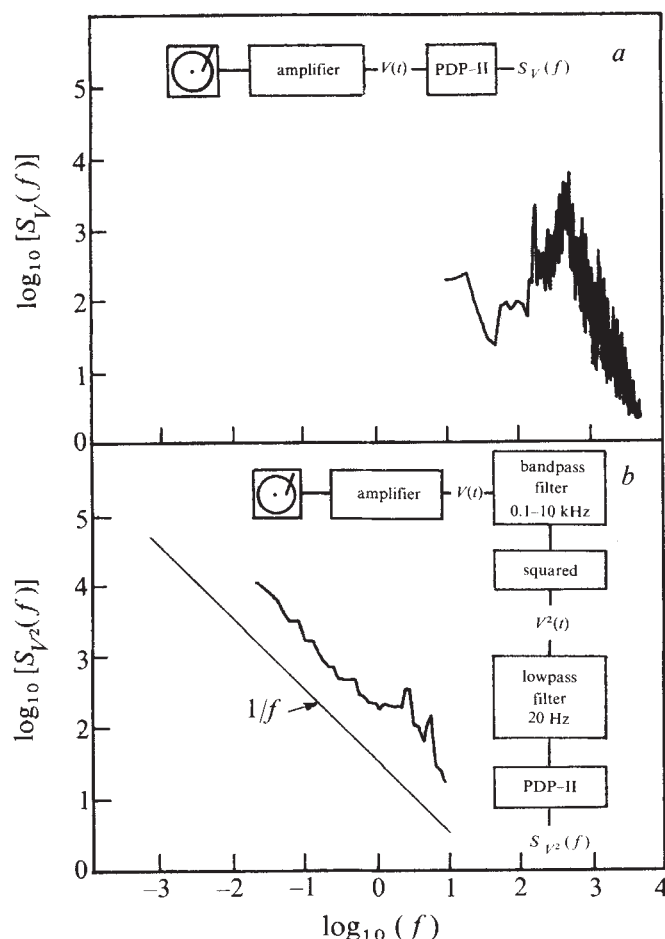


Fig. 1 Bach's 1st Brandenburg Concerto: a, $S_V(f)$ against f ; b, $S_{V^2}(f)$ against f .

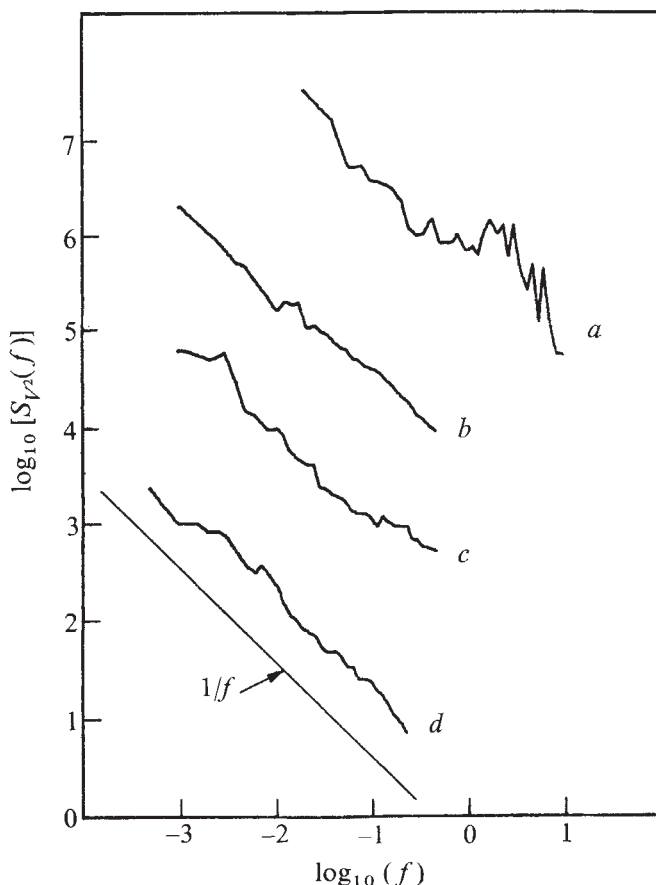


Fig. 2 Loudness fluctuation spectra, $S_{V^2}(f)$ against f for: a, Scott Joplin Piano Rags; b, classical radio station; c, rock station; d, news and talk station.

$|\tau| > \tau_c$. It can be shown that $S(f)$ is 'white' for frequencies $\ll 1/2\pi\tau_c$, and is a rapidly decreasing function of frequency (usually f^{-2}) for frequencies $\gg 1/2\pi\tau_c$. A quantity with a 1/f power spectrum cannot, therefore, be characterised by a single correlation time. In fact, the 1/f power spectrum implies some correlation in $V(t)$ over all time scales corresponding to the frequency range for which $S(f)$ is 1/f-like³. In general, a negative slope for $S(f)$ implies some degree of correlation in $V(t)$ over time scales of roughly $1/2\pi f$. A steep slope implies a higher degree of correlation than a shallow slope.

In our measurements on music and speech, the fluctuating quantity of interest was converted to a voltage whose power spectrum was measured by an interfaced PDP-11 computer using a fast Fourier transform algorithm. Our first measurements were on the output voltage, $V(t)$, of an audio amplifier. Figure 1a shows a log-log plot of the power spectrum, $S_V(f)$, of the audio signal from J. S. Bach's 1st Brandenburg Concerto averaged over the entire concerto. The spectrum consists of a series of sharp peaks in the frequency range 100 Hz–2 kHz corresponding to the individual notes in the concerto and, of course, is not 1/f-like. Although this spectrum contains much useful information, our primary interest is in more slowly varying quantities.

One such quantity is the loudness of the music. The audio signal, $V(t)$, was amplified and passed through a bandpass filter in the range 100 Hz–10 kHz. The filter output was squared and the audio frequencies filtered off to give a slowly varying signal, $V^2(t)$, proportional to the instantaneous loudness of the music. The power spectrum of the loudness fluctuations of the 1st Brandenburg Concerto, $S_{V^2}(f)$, averaged over the concerto is shown in Fig. 1b on a log-log plot. Below 1 Hz the spectrum is 1/f. The peaks between 1 and 10 Hz are due to the rhythmic structure of the music. Figure 2a shows the power spectrum of

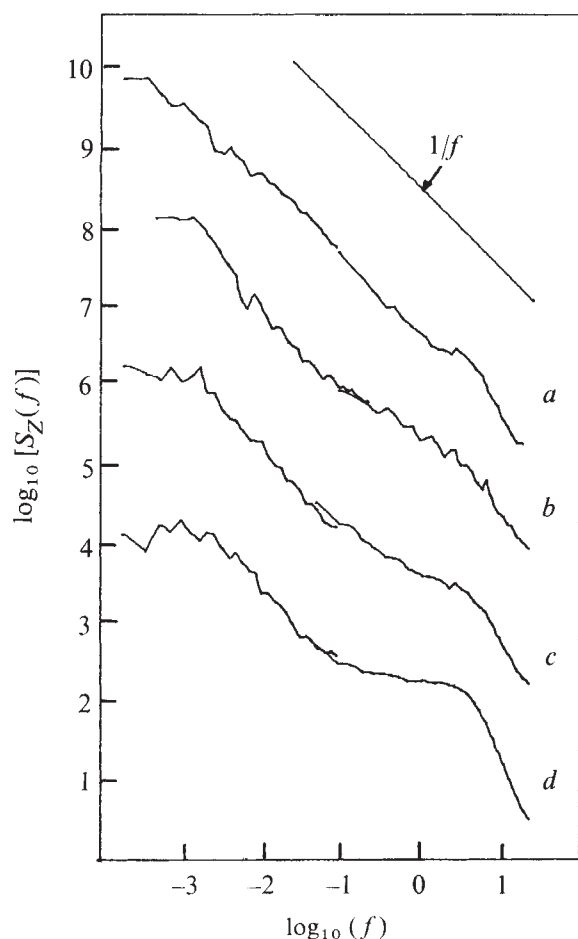


Fig. 3 Power spectra of pitch fluctuations, $S_z(f)$ against f , for four radio stations: *a*, classical; *b*, jazz and blues; *c*, rock; *d*, news and talk.

loudness fluctuations for a recording of Scott Joplin Piano Rags averaged over the whole recording. Although this music has a more pronounced rhythm than the Brandenburg Concerto, and consequently has more structure in the spectrum between 1 and 10 Hz, the spectrum below 1 Hz is still $1/f$ -like.

To measure $S_{V^2}(f)$ down to even lower frequencies, we analysed the output from an a.m. radio. $S_{V^2}(f)$ was averaged over approximately 12 h, and thus included many musical selections as well as announcements and commercials. Figure 2*b-d* shows the loudness fluctuation spectra for three different radio stations. Figure 2*b* shows $S_{V^2}(f)$ for a classical station. The spectrum exhibits a smooth $1/f$ dependence. Figure 2*c* shows $S_{V^2}(f)$ for a rock station. The spectrum is $1/f$ -like above 2×10^{-3} Hz, and flattens for lower frequencies, indicating that the correlation of the loudness fluctuations does not extend over time scales longer than a single selection, roughly 100 s. Figure 2*d* shows $S_{V^2}(f)$ for a news and talk station, and is representative of $S_{V^2}(f)$ for speech. Once again the spectrum is $1/f$ -like. In Fig. 2*b* and *d*, $S_{V^2}(f)$ remains $1/f$ -like down to the lowest frequency measured, 5×10^{-4} Hz, implying correlations over time scales of at least 5 min. In the case of classical music this time is less than the average length of each composition.

Another slowly varying quantity in speech and music is the instantaneous pitch. A convenient means of measuring the pitch is by the rate, Z , of zero crossings of the audio signal, $V(t)$. For the case of music, $Z(t)$ roughly follows the melody. Figure 3 shows the power spectra of the rate of zero crossings, $S_z(f)$, for four radio stations averaged over approximately 12 h. Figure 3*a* shows $S_z(f)$ for a classical station. The power spectrum is closely $1/f$. Figure 3*b* and *c* shows $S_z(f)$ for a jazz and blues station and a rock station. Here the spectra are

$1/f$ -like down to frequencies corresponding to the average selection length, and are flat at lower frequencies. Figure 3*d*, however, which shows $S_z(f)$ for a news and talk station, exhibits a quite different spectrum. The spectrum is that of a quantity characterised by two correlation times: the average length of an individual speech sound, roughly 0.1 s, and the average length of time for which a given announcer talks, about 100 s. Communication, like most human activities, has correlations that extend over all time scales. For most musical selections the communication is through the melody and $S_z(f)$ is $1/f$ -like. On the other hand, for normal English speech the pitches of the individual speech sounds are uncorrelated: the ideas are not directly related to the pitches of syllables but rather to the meanings attached to them. As a result, the pitch power spectrum for speech is 'white' for frequencies less than about 3 Hz, and falls as $1/f^2$ for $f \gtrsim 3$ Hz. In fact, in Fig. 3*a-c* one observes these speech shoulders which become more pronounced as the vocal content of the music increases or the commercial interruptions become more frequent.

The observation of $1/f$ -like power spectra for various musical quantities also has implications for stochastic music composition. Most stochastic compositions are based on a random number generator (white noise source), which produces unrelated notes, or, on a low level Markov process, in which there is correlation over only a few successive notes. Neither of these techniques, however, approximates the $1/f$ spectrum and the long time correlations reported here in music. We have used independent $1/f$ noise sources in a simple algorithm to determine the duration (expressed as half, quarter, or eighth notes) and pitch (expressed in various standard scales) of successive notes of a melody. The music obtained by this method was judged by most listeners to be much more pleasing than that obtained using either a white noise source (which produced music that was 'too random') or a $1/f^2$ noise source (which produced music that was 'too correlated'). Indeed, the sophistication of this ' $1/f$ music' (which was 'just right') extends far beyond what one might expect from such a simple algorithm, suggesting that a ' $1/f$ noise' (perhaps that in nerve membranes⁴) may have an essential role in the creative process.

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Developmental changes in suckling of rat pups

ONTOGENETIC analysis of mammalian suckling provides a unique opportunity to observe the emergence of appetitive behaviour. Transitions in the nature of suckling may reveal progressive stages in the development of adult ingestive systems. Unfortunately, little information is available regarding the nature of suckling behaviour as it develops, and is eventually replaced by feeding and drinking, because it has proved difficult to separate the behaviour of the altricial infant from the contribution of the mother to the nursing dyad. We report here the developmental characteristics of, and changes in, the behaviour of suckling rats as they locate, apprehend and suckle the nipples of anaesthetised dams. This situation permits the detailed evaluation of rat pup behaviour in isolation from the active maternal role.

Even at birth, pups are capable of quickly apprehending and suckling the nipples of an anaesthetised dam. Suckling then undergoes two major transitions. The first, after day 10 *post partum*, suggests the emergence of a physiological control of suckling behaviour. The second, beginning after day 14, indicates a changing responsiveness to some characteristic of the mother's nipples or milk ejection.

Equithesin (Jensen-Salsbery Labs, 2 ml kg⁻¹) was the anaesthetic used. In the conditions reported here (lack of mammary engorgement and the presence of only a few pups), milk let-down with this type of anaesthetic was unlikely. Lack of milk ejection was further confirmed by comparing the weights (nearest 0.1 g) of pups before and after each test. For tests, the anaesthetised dam was laid on her side in a plastic trough (20-cm diameter, in a plastic tub 16 cm × 28 cm × 11 cm) with both nipple lines exposed (the lowest nipple 1 cm from the floor of the trough). Pups were gently placed in groups of three at the mother's side, perpendicular to her flank. The time required for each pup to attach (grasp and swallow) to a nipple was recorded. Also, general activity during the first 30 s was rated on a four-point scale and other behaviours (like rooting) were noted. At every test age, animals were exposed to the mother for three 5-min trials, each separated by a 5-min rest period; mean attachment latency for the three trials was used for analysis. Pups were tested with their own mothers, at several ages; tests of a litter were separated by at least 5 d (a period which was found not to interfere with normal growth). Between tests, pups remained with their mother and were not disturbed. Other pups were tested once only, at various ages, to confirm that specific testing experience did not contribute to attachment behaviour at the later ages.

Pups were tested in deprived and non-deprived conditions. Deprived pups were removed from the litter 22 h before testing, and housed in a warm moist incubator. Non-deprived pups were removed from the mother at anaesthesia. Before, and between, tests axillary temperatures of both groups were kept at litter temperatures (32–36 °C) by placing pups on a warming tray.

Figure 1 presents the mean latencies of deprived and non-deprived pups to attach to the nipples of their anaesthetised mother. Even at the first test ages, pups were able to attach within the 5 min of the tests (41% at 0–1 d).

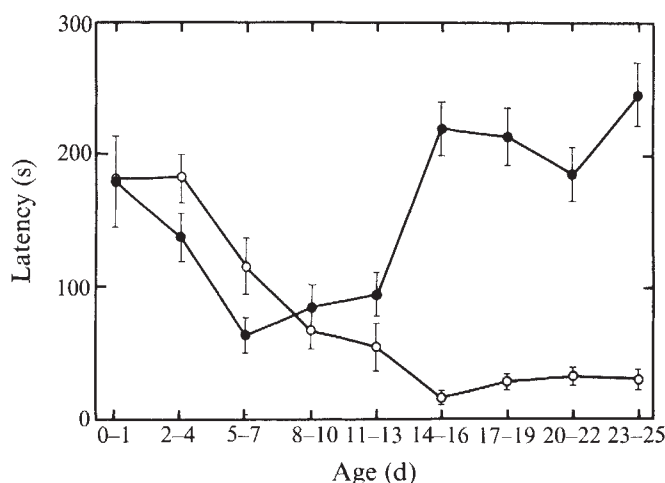


Fig. 1 Mean latency ($\pm$ s.e.m.) of non-deprived (●) and 22-h deprived (○) rat pups to attach to nipples of their anaesthetised mother. Pups at 0–1 d were deprived for only 6 h. A pup's score was taken as the mean of its three 5-min tests. Pups were given a score of 300 s if they failed to attach. Number of subjects varied from 22–36 at each data point, with no more than six pups from the same litter. Pups were tested repeatedly, but not at each data point. The data presented here do not differ from that obtained from pups tested only once.

During the following test intervals (until 8–10 d), the latencies of both deprived and non-deprived pups progressively decreased. This decrease reflects both an increase in the percentage of pups actually attaching, and a decrease in the latency of those that did attach. Attachment latencies for non-deprived pups were actually somewhat shorter than for deprived pups. Once attached, pups remained on the nipple for the duration of the test period despite lack of milk let-down. After 8–10 d, however, a marked transition occurred in the attachment behaviour of non-deprived pups. They shifted from rapid nipple attachment to attachment with long latencies or to non-attachment during the 5-min tests. Thus, before 8–10 d, attachment did not depend on deprivation state; both groups attached quickly and improved their latency to attach. After 8–10 d, attachment was dependent on deprivation (by 14–16 d the difference between deprived and non-deprived pups was statistically significant, $t=8.4$, d.f.=48, $P<0.001$, two-tailed). The change in attachment behaviour was not the result of a parallel change in general activity. Although a difference in activity appeared at 2–4 d, activity of the non-deprived pups remained essentially constant during the transition period [at 8–10 d mean activity rating of 1.8 (± 0.13 s.e.m.), at 17–19 d mean activity of 1.7 (± 0.09)] and by 20 d did not differ from that of deprived pups.

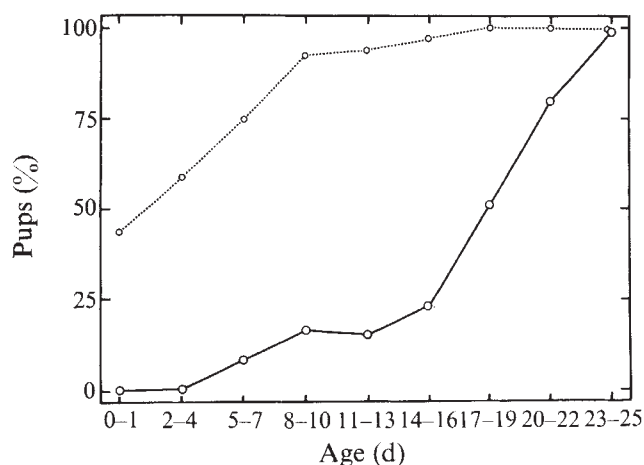


Fig. 2 Percentage of deprived pups shifting nipples—after having attached. This is a mean of the percentage observed in the three 5-min tests. Data are presented only for deprived pups because, by the later ages, very few non-deprived pups attached. The percentage of attachments for these deprived pups is indicated by the dotted curve.

Starting at about day 14 a second change in pup behaviour became obvious. Deprived pups were observed to attach to a nipple, then 20–180 s later would shift to another nipple. The emergence of this behaviour in the sucklings is seen in Fig. 2. Before 14–16 d fewer than 25% of the pups shifted nipples after initial attachment. After 14–16 d, the percentage rapidly increased, until by 23–25 d virtually all pups shifted nipples at least once in each 5-min test. Actually most pups shifted repeatedly. A pup would attach to a nipple, suckle for a few moments and then reject it, only to attach quickly to another.

These findings contribute to a new understanding of aspects of rat suckling behaviour. First, the neonatal rat is capable of attaching and suckling on its own; the mother does not have to play an active role, even at parturition. The array of stimuli, thermal, tactile, and olfactory, that define the mother are sufficient to elicit and sustain suckling. This process in the young rat is independent of immediate milk delivery. In fact, we found that some pups 8 d old and younger will suck for at least 2 h without leaving the nipples of dams anaesthetised with Urethane (Lilly, 1g kg⁻¹).

Second, it is clear that suckling (as defined by the latency to attach to a nipple) is not under the control of nutritional events for the first 8–10 d after birth. Until that time, non-deprived rats apprehend a nipple at least as fast as those deprived of the dam for 22 hr. After that age, however, there is an abrupt change in behaviour, as non-deprived rats take considerably longer to suckle. This change strongly suggests the emergence of a physiological control of suckling.

Third, the development of nipple shifting indicates the nipple of the anaesthetised dam is, after a certain age, no longer an adequate stimulus to sustain suckling in the rat pup. It is possible that pups come to view the nipple as a food source, and then respond in a way to maximise nutritional reward, leaving a nipple that is not producing milk or manipulating many nipples in order to stimulate milk ejection. It has also been noted that pups will leave a nipple just after a milk ejection, when gaining further milk from that nipple is unlikely¹.

Fourth, this preparation, which eliminates the active contribution of the mother from the nursing situation seems to be a reasonable way to isolate and analyse events that control suckling during ontogeny. Drewett, Statham and Wakerly² have recently presented an interesting analysis of the nipple attachments of 10-d-old rat pups hand-held by the experimenter at the nipples of anaesthetised mothers. They found that animals which had been deprived of food were quicker to attach to nipples than were non-deprived pups. Our data extend these findings to a situation where the pup itself is allowed to initiate the search and apprehension of the nipple, and demonstrate a developmental course for this apparent physiological control of suckling.

These data indicate the need for a reassessment of the traditional views of suckling. Suckling behaviour is more than a simple reflex that remains unmodified throughout development. It may prove as complex as the succeeding post-weaning ingestive behaviour of feeding and drinking, and an understanding of its development may contribute to our understanding of adult ingestion.

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Time resolution of acoustic signals by birds

SEVERAL workers have concluded that the response time of the avian ear is of the order of a few ms—far faster than that of the human ear. We have tested the ability of birds to discriminate between a single-click stimulus and a dual-click stimulus and found that this discrimination breaks down when the inter-click interval of the dual click is reduced to a few ms.

The evidence on which the previous conclusions were drawn is as follows. (1) An unsuspected richness of detail emerges when recorded bird vocalisations are played back at slow speeds¹. (2) An analysis of the constancy of reproduction of time intervals in songs of passerines suggests that time discrimination “is no greater than 0.5 ms and may well be considerably less”². (3) The echolocating oil

bird (*Steatornis caripensis*) produces pulses separated by silent intervals of 2–3 ms (ref. 3) and must presumably be able to discriminate echoes within this period. (4) Some birds which sing antiphonally demonstrate a precision of timing down to a few ms (ref. 4). (5) The basilar membrane of the bird ear is comparatively shorter and broader than that of the mammalian ear and the cochlear endolymph seems to be highly damped by the tegmentum vasculosum^{3,6}. This implies that the response time of the avian ear is much shorter than that of the mammalian ear. (6) Electrophysiological responses in the cochlear nucleus of a number of species of birds show nearly 100% phase-locking with clicks reproduced at intervals down to 1–2.5 ms (ref. 7). The time resolution of the auditory neurones in the avian cochlear nucleus is by this measure superior to that of typical mammals.

Much of this evidence must be treated with great caution. Figures for the temporal resolution of the human ear vary greatly according to the method of testing, and should only be compared with those from birds tested in the same conditions. Greenewalt² measured the standard deviations of selected time intervals from repeated phrases of bird song. Equating acoustic performance with auditory discrimination, he suggested that these measures reflected the time discrimination of the avian ear. This would be true only if song production is controlled through auditory feedback. Auditory feedback, while necessary for song learning in some species, is not necessarily essential for the performance of adult song.⁸ The constancy of reproduction of time intervals within adult song may then reflect the precision of ‘read-out’ from a central song template, perhaps influenced by non-auditory feedback loops, and not the time discrimination of the bird’s ear. Further, the temporal patterning of song may, at the lower limits, depend on the response characteristics of syringeal muscles and not on reference to a memory. Responses in single auditory neurones cannot by themselves be taken as an accurate measure of perception. We have therefore sought direct behavioural evidence for temporal discrimination of sounds in birds.

Table 1 Discrimination between dual and single-click stimuli in three species of birds

Subject	Procedure	Discrimination threshold* (ms)
Bullfinch B32	Method of limits	6–3
Bullfinch B34	Method of constant stimuli	4–2
Bullfinch BJ2	Method of constant stimuli	4–2
Bullfinch B39	Method of constant stimuli	4–2
Greenfinch Gr♀2	Method of limits	Below 2
Greenfinch Gr♀3	Method of limits	6–2
Pigeon P♀1	Method of limits	10–2
Pigeon P♂1	Method of limits	10–2

For each subject discrimination between the dual click and a single click was significant ($P < 0.05$) on the first stimulus value but not on the second. The difference between these two values reflects the number of stimulus values on which each subject was tested. Pigeons were tested on fewer stimulus values, and therefore their thresholds determined less precisely, than either the greenfinches or bullfinches.

*Inter-click interval of the dual click.

The discrimination between dual click stimuli and single click stimuli was investigated using operant conditioning. Key pecking was rewarded by access to food. Birds were trained to peck only in the 7.5 s period following a train of dual-click stimuli (inter-click interval for training within the range 100–12 ms, train rate, 1 dual click pers, train duration, 5 s). The period between stimulus presentations was normally 25 s. Stimulus control was increased by having responses in the inter-stimulus period result in a 25 s delay in the onset of the next stimulus presentation. This training was followed by a series of daily 1-h tests in which dual and single click stimuli were randomised.

Responses following dual clicks were again rewarded with food and responses following single clicks resulted in a delay equivalent to that resulting from the period of food access.

The threshold for discrimination between dual and single-click stimuli was defined as the minimum inter-click interval for the dual click on which birds showed discrimination between the dual clicks and the single clicks. This threshold was determined for bullfinches (*Pyrrhula pyrrhula*) by three procedures. In the 'method of limits' the inter-click interval of the dual clicks was progressively decreased over a series of trials from 100 through 50, 25, 12, 6 to 3 ms. The 'method of constant stimuli' differed in that inter-click intervals of 12, 8, 6, 4 and 2 ms were randomised within the experimental series. The discrimination thresholds were found to be similar for both procedures (Table 1).

In the third method, the birds were trained to respond to dual clicks with a particular inter-click interval and the relative responsiveness to these and other clicks was then measured. Figure 1 shows the discrimination gradient

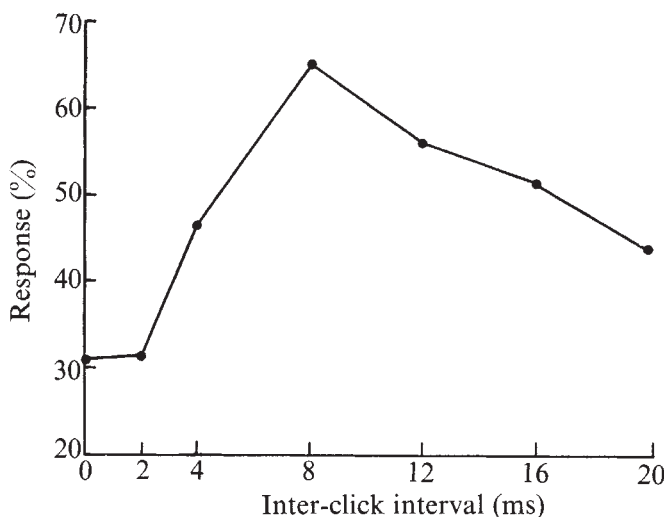


Fig. 1 Discrimination gradient over 15 generalisation tests on bullfinch B54. Each test contained 140 stimulus presentations, 20 on each of seven stimulus values. The click stimuli were presented in random order and the period between presentations was normally 25 s. If one or more key pecks were emitted during a given stimulus presentation then this was scored as a response. The responsiveness to each stimulus value was determined as the number of presentations on which responses occurred expressed as a percentage of the total presentations for that value. Stimulus control was increased by having responses in the inter-stimulus period result in a 25-s delay in the onset of the next stimulus presentation. Each test was preceded and followed by periods in which only stimuli with an inter-click interval of 8 ms were presented. Responses to these stimuli were rewarded with food and the periods were terminated after five rewarded responses. Responding during each test was maintained by rewarding responses to a predetermined five of the 20 stimuli with an inter-click interval of 8 ms.

obtained with this procedure for an adult male bullfinch. Generalisation is complete over the range 2–0 ms (the single click) and the threshold for discrimination of dual clicks from single clicks lies between 4 and 2 ms. This agrees with the results obtained by the other procedures.

We have also tested greenfinches (*Carduelis chloris*) and pigeons (*Columba livia*) and found their discrimination thresholds to be similar to those of the bullfinches (Table 1). Eight human subjects, tested under conditions similar to those experienced by the birds, reported that the two clicks were no longer heard as separate events when the interval between them was reduced to 12–50 ms. Two of the subjects additionally remarked that the composite 'fused' dual click was, however, still discernible from the single click down to inter-click intervals of 2–5 ms. Whether at the lower limits of discrimination the birds hear the

two clicks of the dual-click stimulus as separate events or rely on other possible cues, for example, differences in perceived pitch between a 'fused' dual click and the single click, cannot be determined directly. It is, however, the absolute values for the discrimination thresholds which are important as these define the 'information content' of vocalisations². In any vocalisation only that variation which occurs at suprathreshold level can be used by the bird as information in a communication system.

Rapidly executed frequency and amplitude modulations are common in bird song. The 'sequence calls' of bullfinches contain elements which show modulations with periods of 5–18 ms, and the modulation rates of these elements may be characteristic of particular individuals⁹. The discrimination gradient (Fig. 1) shows that bullfinches are able to discriminate, within the range 2–20 ms, between dual-click stimuli with inter-click intervals differing by only 2 ms. This suggests that information is encoded in the temporal patterning of the cells and is available to these birds. Such information may be of particular importance with regard to individual recognition in bullfinches.

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Inhibition of cortical evoked potentials and sensation by self-initiated movement in man

AN imposed, passive or externally paced displacement (EPD) of the index finger in man evokes brain potentials that differ in delay and waveform for postcentral, precentral and prefrontal areas¹. Displacement so imposed leads to less accurate subjective estimation of limb position from that obtained by self initiated movement². This behavioural observation as far as we know has not been neurophysiologically investigated with cortical recordings from the human brain. Thus we compared the cortical potentials following passive or externally paced displacement, to those related with a similar but self paced voluntary displacement (SPD) of the left index finger. Not only is self-initiated movement manipulative, however, it also subserves acquisition of sensory information. To examine this aspect of finger movement in a second series of experiments we studied the evoked responses and the subjective sensation to brief electrical stimuli applied to the left median nerve at the wrist or the index finger during SPD and compared them with similar responses elicited with the subject at rest.

Multichannel corticographic recordings have been obtained from chronically implanted gold electrodes from six subjects and with similar technique as previously described¹. The finger displacement (of about 45°) was monitored by a movement transducer. The differentiated output of this transducer was used to trigger the PDP 12 computer which was programmed to average the cortical electrical activity preceding and following finger displacement. Somatosensory cortical evoked responses were elicited by electrical stimulation of the left median nerve of the wrist or

the index finger. The stimulator was triggered either by the experimenter with the subject's finger at rest or by a Schmidt trigger activated by the movement during self paced displacement.

Consistently and for all subjects tested the area characteristic response following EPD has diminished, changed in wave form or disappeared completely when the subject himself was moving his finger. In Fig. 1 the evoked potentials from prefrontal, precentral and postcentral cortical areas following EPD can be seen on the left, and the cortical and electromyographic potentials related to similar but self paced displacement on the right. In this as for all subjects tested with SPD no response could be registered from the prefrontal areas, only a broad positivity developed in the postcentral areas and in the precentral areas a diminished amplitude response could be seen in the form of a negative peak.

These changes were not caused by the inherent variability of velocity of each self paced displacement, as averages of selected trials having similar time course showed similar reduction.

A gating action, movement related and exerted on the sensory pathways through their course to the cortex, can explain these results³. This hypothesis has been documented with data from animal experiments³ but, until now, data obtained with scalp electrodes in man have been inconsistent between laboratories⁴.

As was expected in SPD a sustained negativity developed in the motor-sensory areas before the initiation of the movement (Fig. 1). This is the Bereitschaft potential⁵ or N_1 component⁶ known to precede planned action. The negative peak of the precentral area response has been described previously as a "motor potential" or N_2 ⁶. In our records

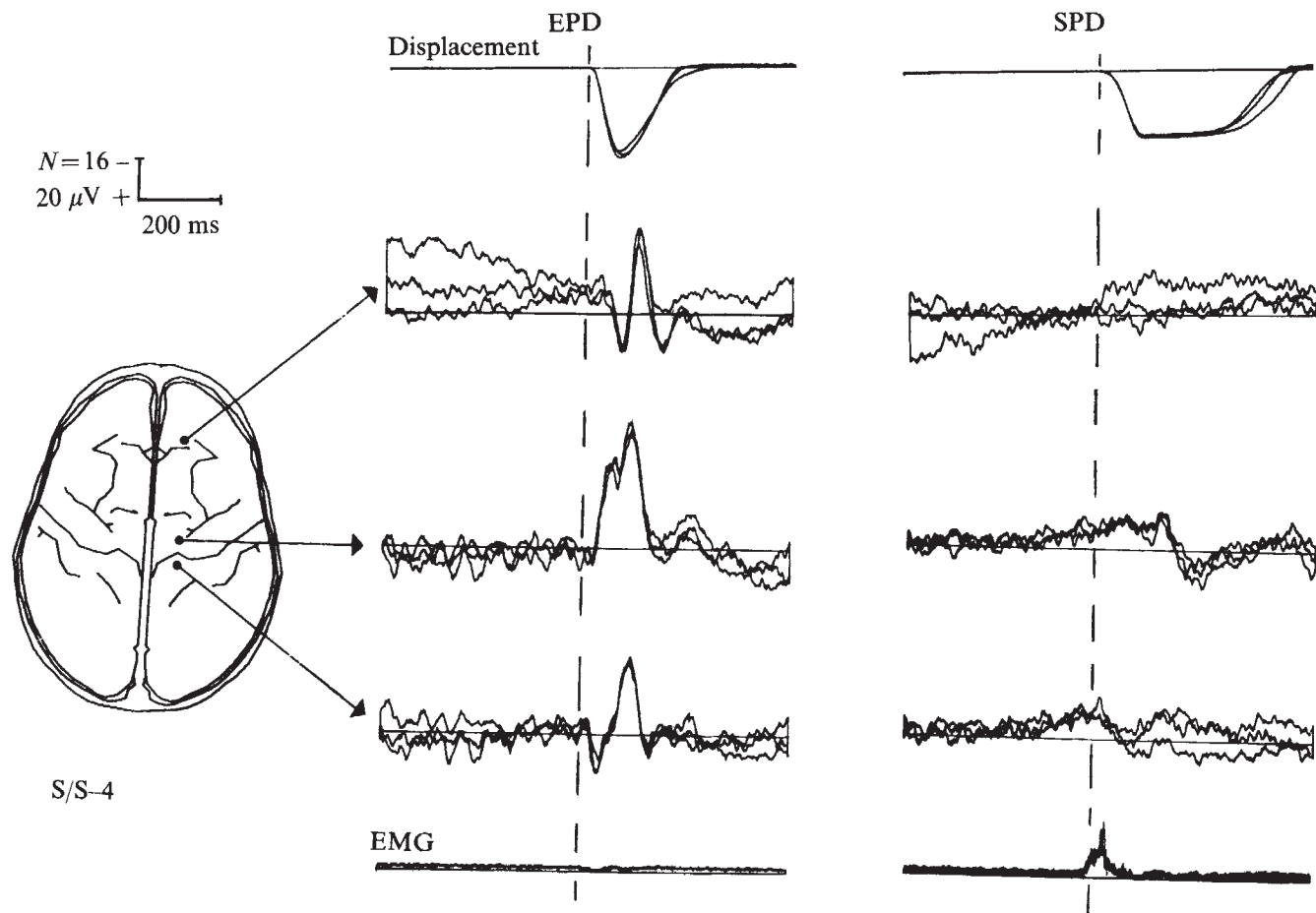
this potential consistently developed only in the precentral cortex contralateral to the movement. Occasionally it preceded the beginning of the movement but never occurred before the beginning of the electromyogram related with the movement. The time from the peak of the electromyogram to the peak of this negativity was between 30 and 110 ms with most values falling between 30 and 50 msec. Contrary to previous suggestions^{5,6} and in agreement with others⁷, and our previous results⁴ we consider this potential, which has been called a "motor potential", to be the first signal of re-afferent activity resulting from the movement. We propose therefore, that N_2 is more appropriately called motor cortex potential (MCP). Our data thus suggest that movement related afferent activity directed towards the precentral cortex is less affected by gating action than when it ascends to other cortical areas.

The selective gating hypothesis has found additional support by studying the somatosensory evoked potentials from pre- and postcentral cortical areas during SPD.

Electrical stimulation of the left median nerve at the wrist evoked clear somatosensory potentials in the pre- and postcentral cortex when the limb was at rest. The wave-forms of these potentials are different⁹ for the two areas (Fig. 2a and c). The potentials evoked by similar stimulation timed to occur during self paced displacement of the left index finger were drastically diminished in amplitude in the postcentral region (d) but remained relatively unaffected at the precentral cortex (b).

These potentials in the postcentral areas were greatly diminished when the stimulus was presented during the first 100 ms of the movement. As the stimulus was further delayed the potentials increased in amplitude reaching their resting values 700 ms from the beginning. This recovery

Fig. 1 Superimposed evoked potentials from prefrontal, precentral and postcentral cortical areas due to left index finger displacement. The position of the recording cortical electrodes at the right hemisphere are indicated as dots in the brain plot. EPD, Responses due to externally paced displacement. SPD, Responses due to self paced displacement. Note that the general suppression during SPD of the potentials following EPD is less pronounced in the precentral cortex (middle cortical traces on the right).



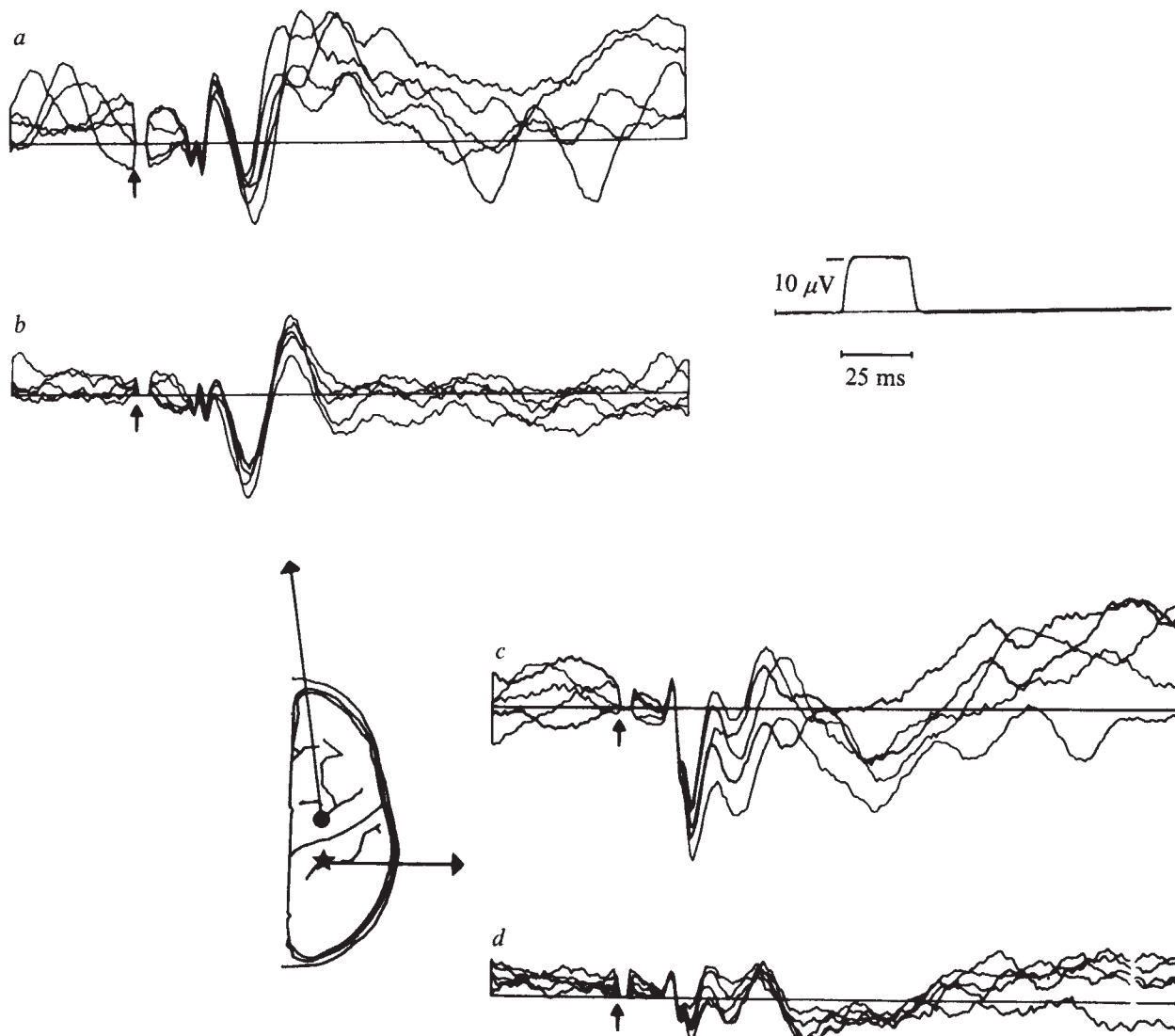


Fig. 2 Superimposed evoked potential to an electrical stimulus applied at the median nerve at left wrist. Traces *c* and *d* from a right postcentral cortical electrode (asterisk in the brain plot). Traces *a* and *b* from a right precentral electrode (dot in the brain plot). Traces *a* and *c* have been simultaneously recorded from the two locations with the subject's finger at rest. Traces *b* and *d* have also been simultaneously recorded during a flexion movement of the left index finger around the metacarpophalangeal joint. Note the differential effect of the movement to the responses of the two areas; the postcentral one has been greatly reduced but the precentral response has retained its amplitude for the majority of the components.

occurred even if the finger was maintained in the displaced position. Both of these observations suggest that peripheral factors are not the cause of the diminished responses¹⁰.

To clarify further the issue of gating and examine its possible behavioural manifestation the median nerve was stimulated and the subject's estimation of the stimulus strength has been determined with the finger at rest or moving. Ten subjects were asked to judge the intensity of three levels of electrical stimulation presented many times to the left index finger in random order when they moved the finger and when it was at rest. The subjective responses showed that all subjects experienced diminished sensation during movement. This diminution occurred only when the stimulated finger was being moved and not when other fingers or limbs were being moved.

Thus by subjective and electrophysiological data a centrifugal gating function is indicated to operate on the afferent activity resulting or occurring in the course of a movement. This gating action selectively affects the input to sensory and motor cortex the second one being less inhibited. Such reduced inhibition for sensory information related to movement and ascending towards the precentral motor cortex is in agreement with the theory of transcortical "reflex loops" proposed on the basis of data collected in animals¹¹ or

from experimentally quantified performance in man¹². The transcortical loop seems to operate through the precentral cortex as it is indicated by both the motor cortex potential (MCP) and the well preserved somatosensory evoked potential from precentral cortex during self-initiated movement. Consequently, the involvement of the primary efferent area in sensory function is at least double. It acts as an input gating mechanism for sensory information towards other cortical areas and as a selector of sensory information upon which prompt action is necessary. To what extent such properties can have clinical significance is too early to say, but it is pertinent to recall that shaking an affected limb is a familiar way of relieving pain after mild injury.

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Methylmercury and selenium in umbilical cords of inhabitants of the Minamata area

It has been established that selenium exerts a protective effect against the toxicity of methylmercury^{1–5}. A study on higher marine mammals suggested that the antagonism between selenium and mercury may result in a marked accumulation of both elements in the organs of the animals⁶, and a similar observation has been made in the laboratory⁷. In retrospect, this beneficial mechanism was operative, though incompletely, during the period when a wide area around the city of Minamata was polluted with methylmercury discharged from a factory involved in the production of acetaldehyde: the organs of men and cats who died from Minamata disease showed elevated levels of selenium as well as mercury^{8,9}. Furthermore, the highest concentration of these substances was noted in the liver of an apparently healthy cat (Hg, 301 p.p.m.; Se, 89.7 p.p.m.) caught in a village 10 miles north of Minamata^{8,9}. We report here that the umbilical cords of the inhabitants of Minamata seem to reflect a chronological transition in

methylmercury pollution but no corresponding shift in selenium levels.

The umbilical cords (~0.3 g) were obtained from the residents of the Minamata area (mostly fishermen) who had kept them in a dried state (a traditional custom in Japan is to use an infusion of the cord as a cure for serious illnesses). A piece (~5 cm long) excised at delivery is wrapped up in gauze and stored in a small wooden box; preservative is added only in exceptional cases. For the analysis of methylmercury we followed the method of Westö¹⁰, selenium was measured by the method of Watkinson¹¹.

The chronological transition of methylmercury and selenium levels in the cords is shown in Fig. 1. Although umbilical cord specimens during the period from 1941–1953 were unavailable, methylmercury levels in the cords seem to reflect two peak periods of acetaldehyde production: a minor peak in the late 1930s and early 1940s and a major one from the late 1950s until 1965. This relationship is more convincingly shown in the series of umbilical cords sampled from two fishery families (Table 1). This observation is in accord with, and expands, a report made by Fujiki *et al.*¹².

Table 1 Methylmercury and selenium concentrations in umbilical cords belonging to the members of two fishery families in Minamata

Family	Year of birth	Sex	Dry weight (p.p.m.)	
			Methylmercury	Selenium
A	1927	F	0.03	0.64
	1929	F	0.04	0.77
	1933	F	0.02	0.63
	1935	M	0.14	—
	1937	F	2.39	0.70
	1939	F	5.87	0.48
B	1953	M	0.04	0.63
	1956	M	2.94	0.76
	1959	F	2.82	0.60
	1962	M	0.22	0.43
	1965	M	0.97	0.63
	1966	M	0.38	0.70

Note that family A reflects the first (minor) peak of acetaldehyde production and the family B the second, and major, peak. The aetiology of Minamata disease was made public in 1960, and consumption of fish from Minamata Bay and the nearby sea greatly diminished, but was not completely stopped. Acetaldehyde production was terminated in 1967.

Two specimens from the late 1930s that showed remarkably high methylmercury levels belonged to the members of a family (family A in Table 1) who lived in a house located very close to the site where waste water from the factory was discharged. The first officially registered case of Minamata disease was put at 1953 in the initial study, but this was later changed to 1942, and the above finding supports the premise that cases of a disease with 'strange' neurological manifestations, noted before, and during those years, many of which were attributed to psychiatric origins, could have been caused by methylmercury¹³. In contrast to previous observations that selenium seems to accompany increased accumulation of mercury or methylmercury in some organs^{6,7,9,14}, this study shows that the selenium levels in the umbilical cords are fairly uniform throughout the observed years regardless of differences in location or time (Fig. 1), and that they are not influenced by methylmercury pollution. This discrepancy between the levels of mercury and selenium has also been noted for human hair (unpublished), and suggests a diversity in the underlying interaction of these elements.

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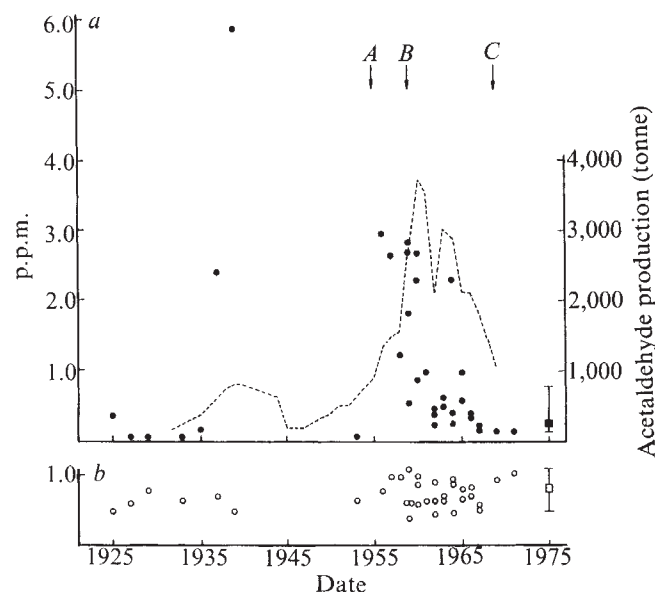


Fig. 1 Chronological transition of methylmercury (a) and selenium (b) levels in umbilical cords, and acetaldehyde production at the Minamata plant. The broken line represents monthly production of acetaldehyde. Methylmercury is a byproduct of this process. Concentrations of selenium and methylmercury are expressed on a dry weight basis. A, Minamata disease publicly recorded. B, Methylmercury identified as causative agent of Minamata disease. C, Production of acetaldehyde terminated. ●, Methylmercury concentration. ○, Selenium concentration. Mean and s.d. ($N = 35$) were calculated as 0.73 ± 0.19 p.p.m. Congenital cases of Minamata disease are not included in this series. There is no particular level of methylmercury in umbilical cords at which a higher incidence of congenital Minamata disease¹⁵. ■, Average concentration of methylmercury in umbilical cords of inhabitants in Tokyo ($N = 24$); Maximum and minimum values are indicated by the bar. □, Average concentration of selenium in the umbilical cords of residents in Tokyo ($N = 24$, mean $\pm$ s.d. = 0.81 ± 0.14 p.p.m.). The bar represents the maximum and minimum values. There was no significant difference between Minamata and Tokyo ($P > 0.5$).

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Investigation of cell lineage in bone using a chimaera of chick and quail embryonic tissue

MOST existing information about the nature of the stem cell(s) in bone that give rise to osteoblasts and osteoclasts is derived from autoradiographs prepared from tissues at various times after the administration of a pulse label of ³H-thymidine¹. These and related studies^{2–5} suggest that a major bone cell precursor population is located immediately around developing bony rudiments and thus close to endosteal and periosteal bone surfaces. Unfortunately, the data obtained by tracing thymidine-labelled cells in bone is usually compromised by two technical problems. First, all cells in the S phase of the cell cycle, whether osteogenic or not, will incorporate ³H-thymidine and appear labelled in autoradiographs. Second, since the isotope is available to dividing cells throughout the body, it is impossible to distinguish between labelled bone cells of local origin and those brought to the site by the blood vascular system. Bone does not represent a closed cellular system.

In the study reported here we followed the lineage of bone cells in a chimaera composed of Japanese quail limb and bone rudiments grown on the chorioallantoic membrane (CAM) of the chicken embryo. This system has the following advantages. First, the CAM provides an immunologically privileged site in which grafts are vascularised by blood vessels and blood cells with an origin different from their own^{6,7}. Second, the distinct morphological differences between the interphase nuclei of the quail and those of the chick allow this organelle to be used as a permanent marker to determine the origin of cells in differentiated tissue at times long after the initial grafting procedure^{8,9}.

The interphase nuclei of most types of quail cells possess one or several large nucleolus-associated heterochromatic masses (Fig. 1a). The interphase nuclei of most chick cells, on the other hand, show a diffuse, somewhat stippled, distribution of heterochromatin (Fig. 1b).

Fertile Japanese quail (*Coturnix coturnix japonica*) and chicken (*Gallus domesticus*) embryos were raised to desired stages of development in a forced draft incubator at 37–38 °C. Embryos of both species were staged using the morphological criteria and numerical designations established for the chick by Hamburger and Hamilton¹⁰. Quail embryos at stages 23–35 (3–8 d incubation) were aseptically

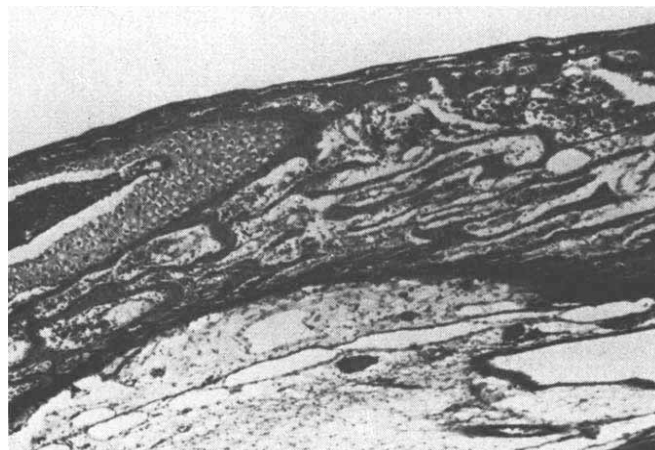


Fig. 2 Photomicrograph showing endosteal, periosteal and endochondral bone formation in a chimaeric graft. Haematoxylin and eosin. Magnification approximately $\times 20$.

removed from their shells and transferred into sterile 0.9% NaCl solution. After removal of the attached extraembryonic membranes using a sterile technique, the fore and hind limbs were amputated. These tissues were then either subdivided into segments 1.5–3.0 mm long, or they were teased apart with watchmaker's forceps to isolate the cartilage models of the bones. The segments and rudiments were then transferred on to well vascularised portions of the CAMs of 7–11 d (stage 34–37) chick embryos through a window cut into the shell¹¹. The graft-bearing eggs were sealed with sterile masking tape and incubated for an additional period of 7–8 d. It should be noted that the percentage of successful takes of cartilage models was significantly increased by placing the rudiments part of the way into a small rend or tear in the CAM. In such instances, the grafts increased 2–3 times in size during incubation and, on occasion, formed structures with strikingly normal external morphologies.

Grafts and control quail and chick tissues were collected

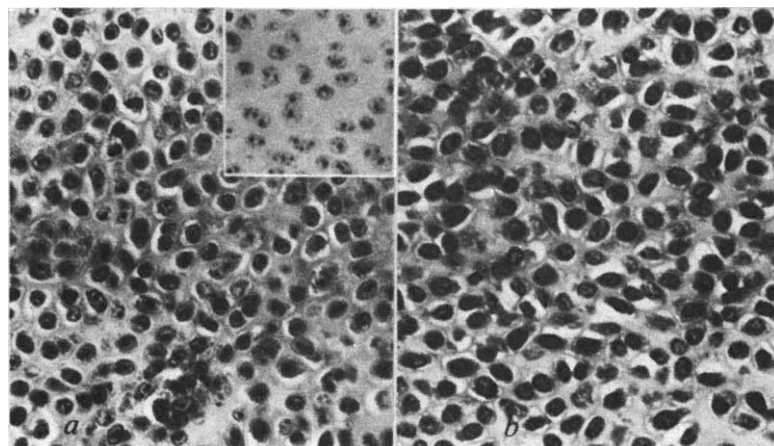


Fig. 1 H and E stained chondrocytes of the quail (a) and chick (b). The heterochromatic masses characteristic of quail interphase nuclei are even more apparent in Feulgen-stained tissue (inset). Magnification approximately $\times 208$.

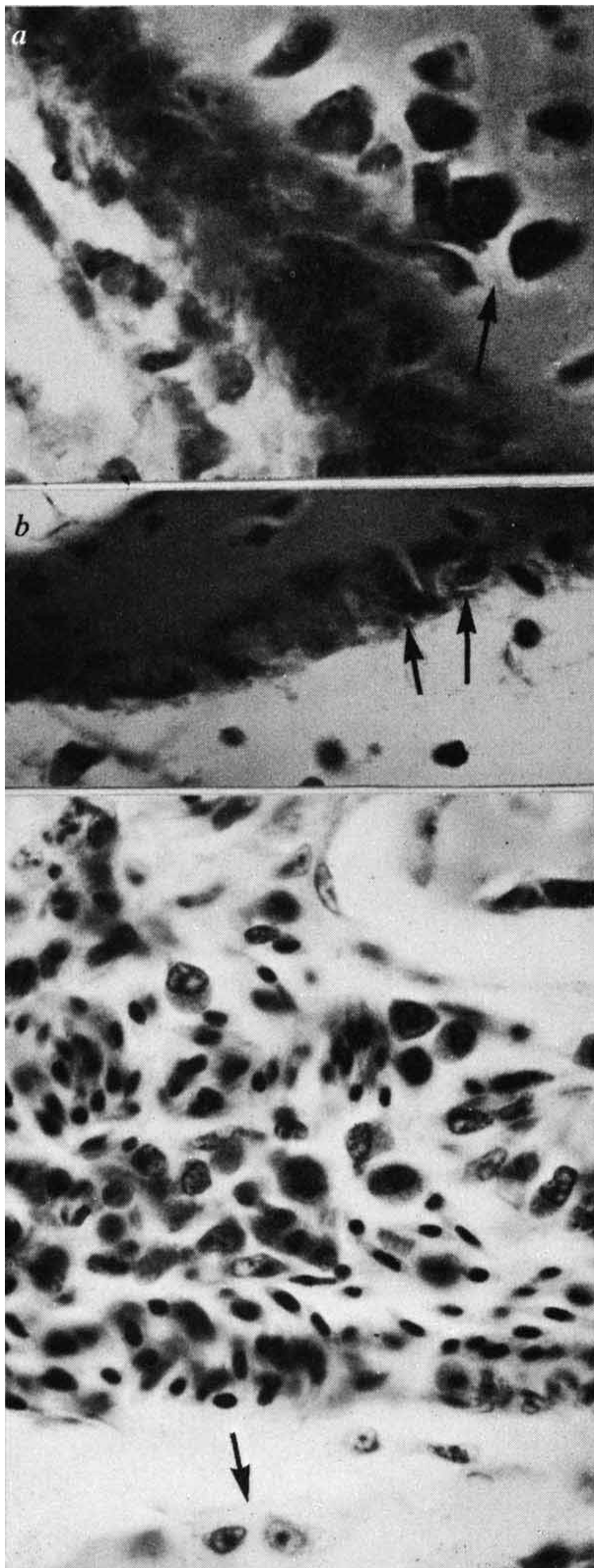


Fig. 3 *a* and *b*, Photomicrographs showing quail-like nuclei in osteoblasts (arrows) lining graft bone surfaces. *c*, The medullary cavity of the chimaeric graft, showing chick-like nuclei in a number of myelopoietic cells. Contrast these nuclei with the quail-like nuclei in the chondrocytes in the lower portion of the field (arrow). All haematoxylin and eosin. Magnifications approximately $\times 750$ (*a*); $\times 300$ (*b*); $\times 750$ (*c*).

and fixed in 10% neutral formalin or Zenker's fluid and embedded in paraffin. Serial sections were then cut at 6–8 μm and stained with haematoxylin and eosin or by the Feulgen method.

Histologically, the grafts show skeletal development that is indistinguishable in major detail from the normal avian pattern. In favourable sections, endosteal and periosteal bone formation, cartilage hypertrophy and erosion, and myelopoiesis within expanding medullary cavities can all be observed (Fig. 2). In addition, some samples also show synovial joints at various stages of development.

Our observations on nuclear morphology suggest an interesting dichotomy in the activities of the chick and

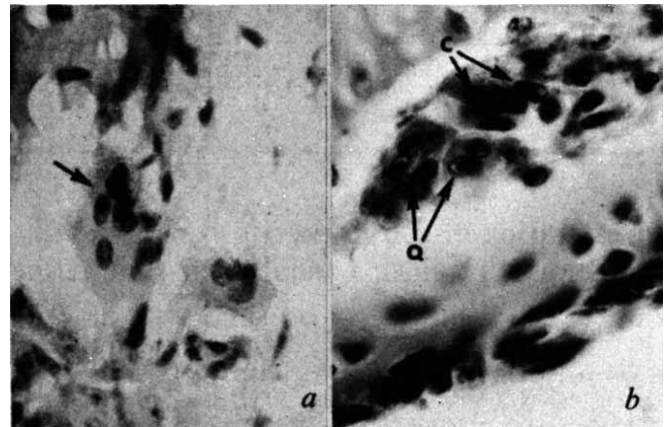


Fig. 4 Photomicrographs showing the nuclear composition of some osteoclasts in chimaeric tissue. Note that there are only two quail-like nuclei (arrow) in the osteoclasts in *a*, whereas in *b* a number of nuclei of both types (Q, quail-like; C, chick-like) can be seen. Feulgen (*a*) and haematoxylin and eosin (*b*). Magnification approximately $\times 470$.

quail cells in the differentiation of the chimaeric graft. Most, if not all, of the cells involved in periosteal and endosteal bone formation have quail-like nuclei (Fig. 3*a* and *b*) indicating a donor (graft) origin for these cells. In contrast, most of the cells in the medullary cavities, including those involved in myelopoiesis, have chick-like nuclei, suggesting a host (haematogenous?) origin (Fig. 3*a*). There were, however, two interesting exceptions. First, cartilage cells in the epiphyses seemed to survive hypertrophy and were conspicuous in the metaphyses adjacent to the eroding cartilage matrix. Whether these cells are in fact viable and whether they can differentiate along osteogenic pathways as suggested by Holtrup¹² and others¹³ is at present under investigation. Second, multinucleated osteoclasts and chondroclasts seemed to be of three types: those with chick-like nuclei, those with quail-like nuclei and those with an admixture of chick and quail-type nuclei (Fig. 4*a* and *b*). The osteoclasts and chondroclasts with chick-like nuclei were the predominant type.

It should be noted that not all the cell types in the chimaeric bones expressed the distinctive species-related pattern of nuclear morphology equally well. The problem was most acute for the cells in the medullary spaces, either because of the heterochromatisation of the nucleus (as in maturing erythrocytes) or because, in some cells, the nucleoli were less compact and distinctive (osteoclasts/chondroclasts, myelocytes). The species origin of osteoblasts, osteocytes, chondrocytes and fibroblasts could almost always be established with confidence in well fixed and well stained preparations.

These observations support the concept that most osteoclasts are derived from mononucleated haematogenous cells such as phagocytes and monocytes^{15–20}. They also confirm^{4,21,22} that some osteoclasts arise from the "fusion" of bone cells *in situ*. We found no evidence, however, that

osteoclasts can disaggregate to form osteoblasts directly or indirectly through dedifferentiation to precursor cells. The percentage of osteocytes in both chimaeric and native quail bone that had quail-like nuclei was identical (~90%). It would seem, therefore, that few (blood-borne) chick-type osteoclast nuclei in this bone modelling system have the capacity to form osteogenic cells. The survival and/or death of osteoclast and chondroclastic cells in bone thus remains an open question. Confirmatory studies using tissues labelled with ^3H -thymidine and immunohistology are in progress.

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Stimulation of *in vitro* somite chondrogenesis by procollagen and collagen

THE embryonic notochord stimulates or induces the formation of vertebral cartilage by embryonic somites^{1,2}. Evidence indicates that proteoglycans produced by the notochord are involved in mediating the conversion of somitic sclerotomal cells into cartilage. During its interaction with somites, the notochord synthesises and secretes proteoglycans (and collagen) which initially accumulate in the perinotochordal sheath and subsequently diffuse among adjacent somitic sclerotomal cells just before the differentiation of the cells into cartilage³. Extracellular proteoglycan aggregate (consisting predominantly of proteochondroitin 4- and 6-sulphate) greatly stimulates *in vitro* somite chondrogenesis in the absence of notochord⁴. Notochords from which proteoglycans of the perinotochordal sheath have been selectively removed are impaired in their ability to support chondrogenesis but reacquire their ability to stimulate chondrogenesis concomitant with their reacquisition of perinotochordal proteoglycans^{5,6}.

Although these results implicate proteoglycans produced by the notochord in the control of somite chondrogenesis, several observations suggest that collagen produced by the notochord may also be involved⁷. Collagen synthesised by the notochord consists only of $\alpha 1$ chains and seems to have an amino acid composition identical to type II cartilage collagen^{7,8}. In addition, notochords from which both the collagen and proteoglycans of the perinotochordal sheath have been removed are more impaired in their

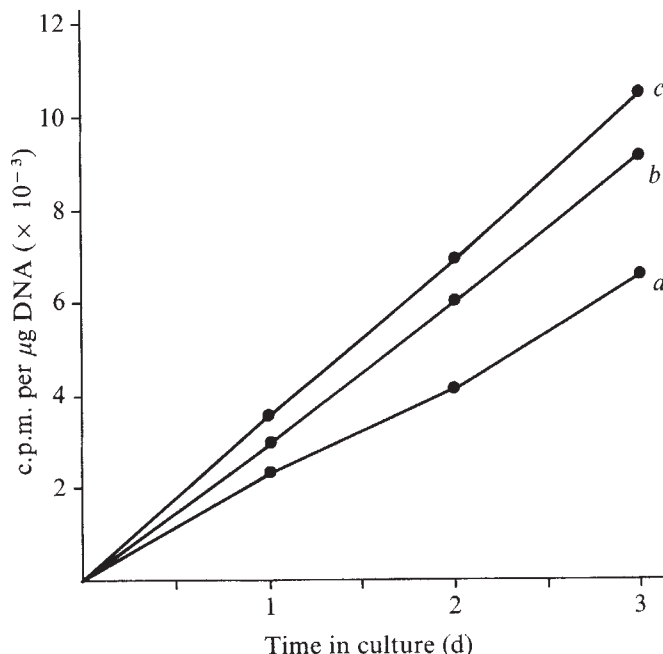


Fig. 1 Accumulation of ^{35}S -sulphate-labelled glycosaminoglycans (GAG) by control somite explants (a) and by explants supplied with exogenous type I (b) and teratoma (c) procollagen. GAGs were extracted from somite explants continuously exposed to $5.0 \mu\text{Ci ml}^{-1}$ of $\text{H}_2^{35}\text{SO}_4$ (carrier free, New England Nuclear) described in ref. 5. DNA was determined by a micro-modification of the procedures of Abraham *et al.*²¹ and Richards²². The experiment was repeated five times and the accumulation of ^{35}S -sulphate-labelled GAG by explants in the presence of type I procollagen ranged between 30% and 50% above the controls by the third day of culture, whereas the accumulation in the presence of teratoma procollagen ranged between 60 and 65% above the controls.

ability to support chondrogenesis than notochords from which proteoglycans have been selectively removed^{5,6}. Finally, collagen, as well as proteoglycans, has been directly implicated in one other tissue interaction, that between lens and corneal epithelium^{9,10}.

We have investigated the effect of collagen on *in vitro* somite chondrogenesis. In addition, since it has been established that collagen is initially synthesised and secreted in a soluble precursor form (procollagen), it was of interest to test the effect of procollagen. Further, the extensive solubility of procollagen in physiological conditions enabled us to compare different procollagen types at equivalent concentrations on their ability to stimulate chondrogenesis. We have found that several distinct molecular species of procollagen and collagen do indeed stimulate *in vitro* somite chondrogenesis. Further, our results suggest that certain types of procollagen and collagen are more effective than others in promoting chondrogenesis. Particularly effective in promoting chondrogenesis is type II collagen, which is the type of collagen synthesised and secreted by the notochord during its interaction with somites.

Somites were dissected from stage 17 embryos¹¹ of White Leghorn chicks (see ref. 12). Explants consisting of 8–10 somites were cultured on nutrient agar¹³ and fed with F12X medium containing 20% foetal calf serum, and were examined daily for visual signs of chondrogenesis (see ref. 13). To determine the effect of exogenous procollagen on chondrogenesis, the feeding medium was supplemented with $200 \mu\text{g ml}^{-1}$ of procollagen. Type I procollagen was purified from the medium of cultured human diploid skin fibroblasts (see ref. 14). These molecules have a chain composition of $[\text{Pro } \alpha 1(\text{I})_2 \text{ Pro } \alpha 2]$ and demonstrate an amino acid composition comparable to that of type I procollagens purified from other sources¹⁵. Procollagen was also purified from the medium of a clonal line of mouse teratoma cells.

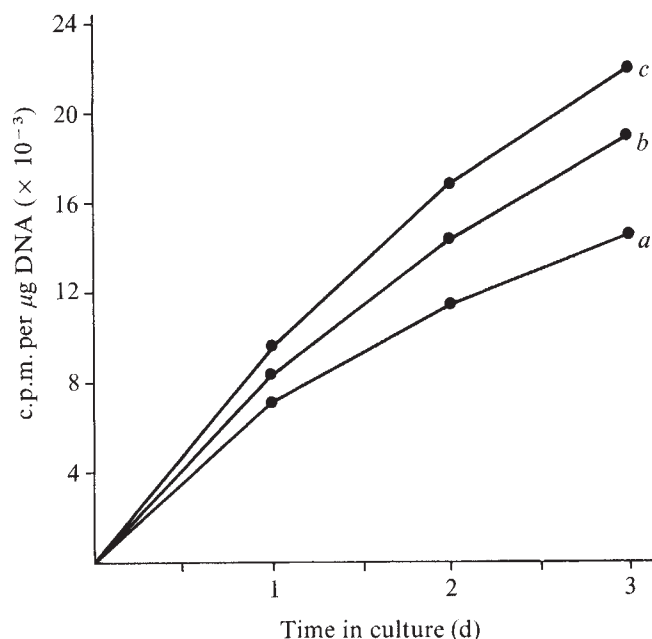


Fig. 2 Accumulation of ^{14}C -glycine-labelled collagen by control somite explants (a) and by explants supplied with exogenous type I (b) and teratoma (c) procollagen. Collagen synthesis was assayed by the incorporation of ^{14}C -glycine into collagenase-sensitive materials utilising a protease-free collagenase (see refs 19 and 20). The experiment was repeated three times and the accumulation of ^{14}C -glycine-labelled collagen by explants in the presence of type I procollagen ranged between 30 and 35% above the controls by the third day of culture, whereas the accumulation in response to teratoma procollagen ranged between 46 and 55% above the controls.

This teratoma procollagen seems to be of a unique type since it consists only of Pro $\alpha 1$ chains and has an amino acid composition that is distinctly different from all other known collagens and procollagens having only $\alpha 1$ or Pro $\alpha 1$ chains in the molecule (R. L. Church, in preparation). In addition, somite explants were cultured on Millipore filters (type HA, 0.45 μm pore size) or on Millipore filters on which collagen substrates were prepared. Collagen substrates were made by preparing collagen gels on Millipore filters (see ref. 16) and concentrating the collagen in the gels by drying (see ref. 9). Purified type II collagen from lathyrus chick sternal cartilage was prepared by differential salt fractionation¹⁷. Purified type I collagen from calf skin was prepared as described in ref. 18.

Repeated visual observations indicated that in the presence of exogenous Type I or teratoma procollagen there was a consistent increase in the amount of hyaline cartilage matrix formed by somites. To confirm these visual observations of enhanced chondrogenesis, we studied the accumulation of sulphated glycosaminoglycans (GAG) and collagen, the major constituents of cartilage matrix, in response to exogenous procollagen.

The accumulation of ^{35}S -sulphate-labelled GAG by somite explants cultured in medium supplemented with type I and teratoma procollagen is shown in Fig. 1. By the third day of culture, somites cultured in the presence of type I procollagen accumulated approximately 40% more sulphated GAG than controls. This experiment has been repeated five times and the accumulation of sulphated GAG by explants in the presence of extracellular type I procollagen ranged between 30% and 50% above controls. In contrast, somites cultured in the presence of extracellular teratoma procollagen consistently accumulated 60–65% more sulphated GAG than controls (Fig. 1). These results indicate that although both types of procollagen are stimulatory, teratoma procollagen is more effective than type I.

Figure 2 demonstrates the accumulation of ^{14}C -glycine-

labelled collagen by somites in response to extracellular Type I and teratoma procollagen. Explants in the presence of type I consistently accumulated 30–35% more collagen than the controls, whereas those in the presence of teratoma procollagen consistently accumulated approximately 50% more collagen than the controls.

These results indicate that both types of procollagen stimulate sulphated GAG and collagen accumulation by somites, an observation that correlates well with the consistent increase in the amount of cartilage matrix formed in response to each type of procollagen. Further, they indicate that although both types of procollagen are stimulatory, teratoma procollagen is a more effective stimulator than type I. The stimulatory effect of procollagen on sulphated GAG and collagen accumulation by somite explants seems to be specific for these matrix components, since type I procollagen had no effect on the incorporation of ^{14}C -glycine into non-collagen protein, and teratoma procollagen increased the accumulation of non-collagen protein by only 5%. In addition, only 4–5% of the total labelled GAG and collagen accumulated by explants supplemented with type I or teratoma procollagen was found in the medium of the cultures, whereas 8–9% of the total labelled material was found in the medium of the control explants.

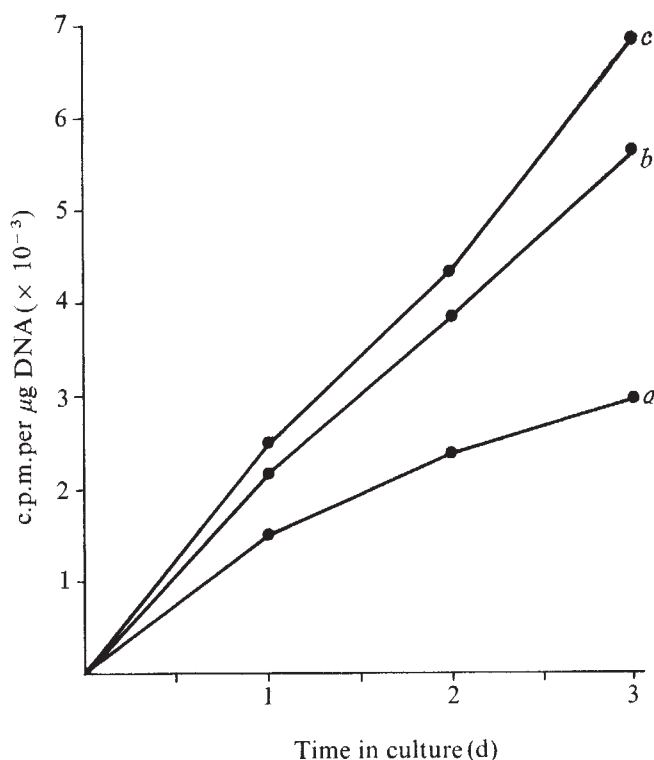


Fig. 3 Accumulation of ^{35}S -sulphate-labelled glycosaminoglycans (GAG) by control somite explants cultured on Millipore filters (a) and by explants cultured on type I (b) or type II (c) collagen substrates. The experiment was repeated four times and the accumulation of ^{35}S -sulphate-labelled GAG by explants cultured on type I collagen substrates ranged between 1.95 and 2.05 times greater than the controls by the third day of culture, whereas the accumulation on type II substrates ranged between 2.4 and 2.5 times greater than the controls.

This suggests that procollagen not only stimulates the total accumulation of matrix components by somite explants, but that it also enhances the retention of these components by the tissue. Finally, neither type I nor teratoma procollagen seems to enhance the survival or viability of somite explants, since there is no difference in the total DNA content of control or experimental explants during any period of culture.

The response of somites cultured on purified type I or type II collagen substrates prepared on Millipore filters was

compared with the response of somites cultured on Millipore filters alone or on Millipore filters coated with bovine serum albumin.

On collagen substrates there was a striking increase in the amount of hyaline cartilage matrix detectable visually with the dissecting microscope and histologically. A detailed light microscopic and ultrastructural analysis of the enhanced chondrogenic response of somites to collagen substrates will be published elsewhere. The visual observations correlate well with the biochemical response of somites to collagen substrates as shown in Figs 3 and 4.

Figure 3 compares the accumulation of ^{35}S -sulphate-labelled GAG by somites cultured on type I and II collagen substrates with the accumulation by controls cultured on Millipore filters. By the third day of culture, there was a consistent twofold increase in the amount of sulphated GAG accumulated on type I substrates and a 2.4- to 2.5-fold stimulation of type II substrates.

Similarly, approximately 75% more ^3H -proline-labelled collagen was accumulated by somites on type I substrates than by controls, and twice as much collagen was accumulated on type II substrates (Fig. 4).

The enhanced accumulation of the components of cartilage matrix by somites on collagen substrates seems to be relatively specific, since the accumulation of ^3H -proline-labelled non-collagen protein is increased by only 24% on type I substrates and by only 31% on type II substrates. The slightly enhanced accumulation of non-collagen protein on collagen substrates undoubtedly reflects to some extent the enhanced accumulation of the protein core of sulphated GAG (proteoglycan). Further, there is an enhanced retention of matrix components by the tissue on collagen substrates, since only approximately 4% of the total labelled GAG and collagen accumulated by explants on collagen substrates is liberated to the medium, whereas approximately 10% is found in the medium of control cultures. In addition, collagen substrates do not affect the viability of explants, since there is no difference in the total DNA content of control and experimental explants during any period of culture.

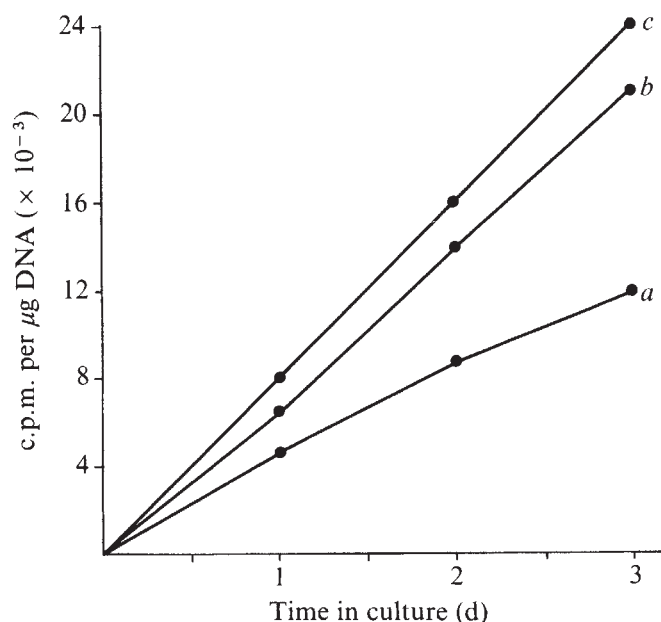


Fig. 4 Accumulation of ^3H -proline-labelled collagen by control somite explants cultured on Millipore filters (a) and by explants cultured on type I (b) or type II (c) collagen substrates. The experiment was repeated four times and the accumulation of ^3H -proline-labelled collagen by explants cultured on type I collagen substrates ranged between 1.75 and 1.81 times greater than the controls by the third day of culture, whereas the accumulation on type II substrates ranged between 1.95 and 2.07 times greater than the controls.

We were unable to test the effect of type II procollagen on somite chondrogenesis, since we do not have an adequate supply of purified Type II procollagen. That is, however, an important experiment for future investigation as the indications are that type II is the type of collagen synthesised and secreted by the notochord during its interaction with somites^{7,8}.

We did, however, find that purified type II collagen substrates caused a striking stimulation of *in vitro* somite chondrogenesis. Furthermore, type II substrates were consistently more effective in promoting chondrogenesis than type I collagen substrates. We cannot, however, be certain that somites have access to equivalent amounts of collagen on each type of substrate. Although we have made every effort to ensure that the substrates of the two types of collagen were prepared from collagen solutions of equivalent concentrations, it is difficult to control accurately the amount of collagen interacting with tissues using insoluble collagen substrates, because of potential differences in the polymerisation properties of the two types of collagen or potential differences in the thickness of the two substrates⁹. Nevertheless, the fact that we have never done an experiment in which type II collagen did not cause a greater stimulation than type I suggests that type II collagen may indeed be more effective than type I in promoting chondrogenesis. We should be able to answer this question about collagen type specificity more clearly when we have examined the effect of soluble type II procollagen on chondrogenesis.

An important consideration in these experiments is the purity of the procollagen and collagen preparations used to promote chondrogenesis. Although it is difficult to rule out absolutely the possibility that a trace amount of non-collagen material is present in any purified collagen preparation, the purity of all of the procollagen and collagen preparations used in these experiments has been established by a number of rigid criteria^{14,17,18}.

The mechanism by which extracellular matrix materials stimulate somite chondrogenesis is not known. It has been suggested that extracellular proteoglycans exert their effect on somite chondrogenesis by interacting with components of the somite cell surface^{4,5}. Similarly, we propose that a likely site of action of collagen is the somite cell surface. The fact that completely insoluble collagen substrates stimulate chondrogenesis is strong evidence that collagen is exerting its regulatory effect by interacting with the cell surface.

Regardless of its mechanism of action, the results of the present investigation indicate that collagen, as well as proteoglycans, is involved in the control of somite chondrogenesis and that these two classes of extracellular matrix materials may synergistically mediate the conversion of somite sclerotomal cells into cartilage.

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Early-labelled haem in erythroid and hepatic cells

SEVERAL lines of evidence point to the existence of a rapidly synthesised haem fraction which is unrelated to any of the known haemoproteins. Much of this evidence has come from studies of the early-labelled fraction of bilirubin which, unlike most bilirubin produced in normal conditions, is derived from sources other than haemoglobin in senescent red cells^{1,2}. As measured with ¹⁴C-glycine, the early bilirubin fraction consists of both a rapid and a slow component^{3,4}. In rats in normal conditions, the slow component seems to be derived chiefly from haemoproteins with different rates of turnover^{5,6}. The metabolic source of the initial sharp component remains more enigmatic. The rapidity of its formation, only 1–2 h after ¹⁴C-glycine administration³, does not conform to the turnover rate of any known haemoprotein. Here we describe a rapid peak of haem labelling, just preceding the initial bilirubin component, in both liver and young erythroid cells, suggesting that rapidly synthesised haem may be of importance in all the major sites of haem production.

2-¹⁴C-glycine (18 mCi mmol⁻¹, New England Nuclear), 40 μ Ci per 100 g body weight, was given in single intravenous injections to male Sprague–Dawley rats weighing 150–250 g. Animals were fasted for 17 h before the isotope was given. At 0.5, 1, 1.5, 2.5, 4.5, 8, 16, 48 and 96 h, groups of 4–6 rats were exsanguinated by cardiac puncture and red cell haemin isolated for radioassay³. Livers were perfused with cold physiological saline, excised, and homogenised in 4 volumes of cold 0.25-M sucrose. A portion of the homogenate was sedimented into mitochondrial and microsomal fractions⁷. Bone marrow cells were flushed from both tibias, pressed through stainless steel mesh and washed three times with phosphate-buffered saline. Measured amounts of non-radioactive rat haemoglobin were added to the bone marrow suspensions and the liver fractions, 1% deoxycholate was added as solubiliser, and haemin was crystallised for determination of specific activity³. This method yielded 70, 65 and 60% recoveries of ¹⁴C-haemin from whole liver, mitochondrial and microsomal fractions respectively, as determined from the radioactivity in livers of animals given ¹⁴C-haemin intravenously 5 min before they were killed. Reproducibility of ¹⁴C-haemin isolation was within 1%.

There were two early peaks of ¹⁴C-haem labelling in the liver (Fig. 1). The first was a sharp peak which was maximal 1 h after the administration of ¹⁴C-glycine and the second was a more gradual peak maximal at 4.5 h. Qualitatively similar patterns were observed in both the mitochondrial and microsomal fractions, although glycine incorporation into haem was consistently somewhat higher in the latter than in the former. On the basis of assays of NADPH-cytochrome c reductase⁸ and cytochrome oxidase⁷ to determine the recovery of microsomes and mitochondria respectively from the total liver homogenate, it is estimated

that the ¹⁴C-haem present in both these fractions accounted for 50% of total hepatic haem labelling at 1 h, as compared with 73% at 4.5 h. Studies in rats given ⁵¹Cr-labelled red cells indicated that hepatic blood contributed negligibly to ¹⁴C-haemin isolated from the liver. There was no histological evidence of extramedullary erythropoiesis in the liver.

Rapid sharp peaks of ¹⁴C-haem labelling were also observed in cells from both peripheral blood and bone marrow (Fig. 2). As with the liver, these were maximal 1 h after glycine was given. Both peaks were virtually abolished in rats hypertransfused with volumes of packed red cells equivalent to 3.2% body weight on both the sixth and second days before glycine injection, indicating their origin from immature erythroid cells. The marrow peak was higher than that in peripheral blood. After 1 h marrow haem labelling fell sharply and then increased quickly again, as anticipated for effective haemoglobin synthesis. There was also an abrupt fall in haem labelling in the peripheral blood samples after 1 h. Thereafter, labelling increased gradually over the 96 h of observation, as anticipated from earlier studies³.

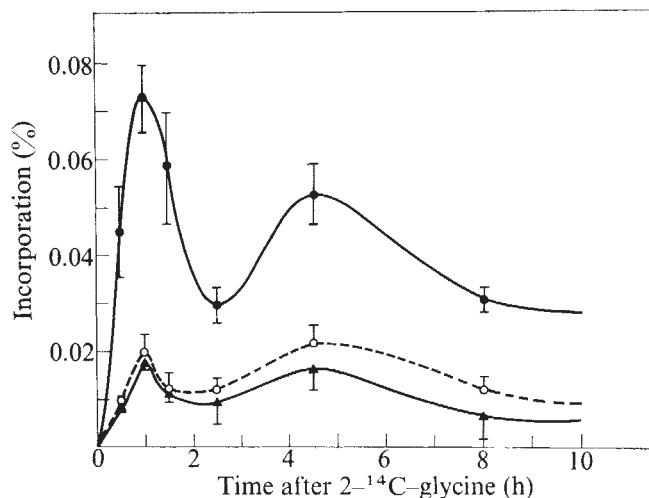


Fig. 1 Incorporation of 2-¹⁴C-glycine into liver haem. Each point represents mean $\pm$ s.e.m. for 4–6 rats. After 8 h, labelling continued to fall gradually, reaching a stable level between 48 and 96 h. Values for mitochondria ($\blacktriangle$) and microsomes ($\circ$) are corrected for the efficiency of recovery of these fractions from whole liver ($\bullet$), based on assays of cytochrome oxidase and NADPH-cytochrome c reductase respectively.

A rapid peak of haem labelling in liver has been reported previously⁹. The second component observed in this study was not found, however, perhaps because precursors such as δ -aminolaevulinic acid were used instead of ¹⁴C-glycine; the present study was based on the assumption that glycine provides a more physiological representation of haem synthesis³. Because of their similar kinetics³, it is probable that this second phase of hepatic haem labelling is a source of the second phase of early bilirubin production, supporting the conclusion that this bilirubin component arises largely from non-haemoglobin haems in normal rats³.

Most of the evidence to date has indicated that the initial component of early-labelled bilirubin originates in the liver^{9–12}. The demonstration of rapid peaks of haem labelling not only in liver, but also in young erythroid cells, suggests that all these tissues give rise to the rapid 'non-erythroid' fraction of bile pigment. This may explain the hitherto puzzling observation that this bilirubin fraction may be increased in conditions of marked erythroid stimulation^{13,14}. In fact, the rapid fall in haem labelling after 1 h in liver, marrow and blood significantly exceeds the formation of labelled bilirubin at this time³, suggest-

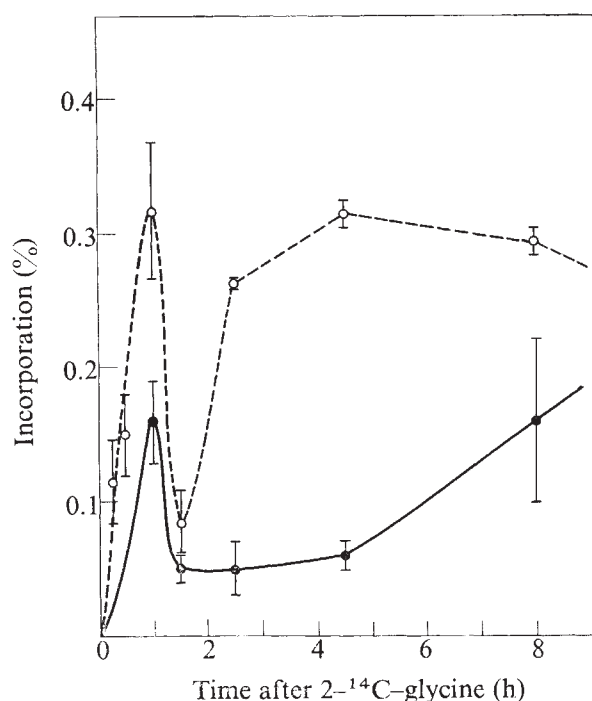


Fig. 2 Incorporation of $2\text{-}^{14}\text{C}$ -glycine into haem in peripheral blood and bone marrow cells. Each point represents mean $\pm$ s.e.m. for 4–6 rats. After 8 h, haem labelling in marrow cells (○) decreased gradually, reaching a stable level of 0.06% between 48–96 h, while labelling in peripheral blood (●) continued to rise, reaching a value of 0.58% at 96 h. The fraction of the total marrow represented by the assayed tibial cell suspensions was estimated to be $1.3 \pm 0.074\%$ as follows: ^{59}Fe in tibial cells collected after maximal plasma ^{59}Fe disappearance at 5 h (4 rats) was compared with maximal ^{59}Fe incorporation into red cells at 4 d (4 rats). This assumes that all ^{59}Fe present in marrow at 5 h would have been recovered in peripheral red cells at 4 d, and probably leads to some underestimation of total marrow mass.

ing that some of the early-labelled haem is degraded to products other than bilirubin^{9,15} or that some haem is not catabolised but is altered temporarily to a less extractable form.

The presence of rapid peaks of haem labelling in both of the major sites of haem synthesis—erythroid and hepatic cells—suggests that haem with a rapid rate of turnover is characteristic of all tissues in which haem synthesis takes place. The similar kinetics of the peaks in these different tissues suggest that they all have the same metabolic origin, perhaps a pool of free or unassigned haem, as has been proposed for the liver⁵. There is evidence for some free haem in rabbit reticulocytes¹⁶ and in nucleated erythroid cells from mice¹⁷. Free haem could serve several functions: to act as a prosthetic group for different haemoproteins, to help govern the rate of overall haem synthesis¹⁸, and to regulate the synthesis of proteins both related and unrelated to haem as a prosthetic group¹⁹.

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Inversion of levels of human T and B cells in early pregnancy

CELL-MEDIATED immunity has been shown to be decreased during pregnancy^{1,2}. Finn *et al.*³ suggested that the cause may be a decrease in the number of T cells; however, levels of T cells were unchanged in women in the third trimester of pregnancy⁴. In addition, the normal number of B cells was found in women who had been pregnant for at least 6 months⁵. Gergely *et al.*⁶ also found normal levels of T and B lymphocytes in women between 34 and 36 weeks of gestation. So far no change has been shown in T- and B-cell levels during the latter half of pregnancy.

Here we confirm the earlier results and present evidence for an inversion of the percentage of peripheral blood T and B lymphocytes during early pregnancy. This inversion is evidenced by a relative drop in the percentage of T lymphocytes coupled with an increase in B lymphocytes during the first trimester. These altered T- and B-cell ratios revert to normal during the second trimester and remain normal throughout the rest of pregnancy.

The percentage of T and B cells in Ficoll-Hypaque-purified peripheral blood lymphocytes was determined using direct and indirect rosette techniques⁷. B cells (Ig^+T^-) were identified by rosetting with mixtures of human erythrocytes coated with anti-human κ light chain antibody or anti- λ light chain antibody. T cells (Ig^-T^+) were indirectly rosetted by first sensitising lymphocytes with specific rabbit anti-human T cell antisera. After thoroughly washing these sensitised cells with RPMI 1640 (Grand Island Biological) the T^+ cells were rosetted with human erythrocytes coated with anti-rabbit light chain antibodies.

The percentages of peripheral blood T and B cells were determined for women at different times during pregnancy and compared with average T- and B-cell percentages of four women who were not pregnant (Table 1). From the 7th to 20th week of pregnancy, the percentage of T cells was much lower and the percentage of B cells much higher than in the non-pregnant women (Table 1). On the other hand, from the 22nd week until term, the percentages of B and T cells were essentially the same as in non-pregnant women (Table 1). The change in percentage of B and T cells could result from a unilateral decrease in T cells or an increase in B cells or both. A unilateral change would be reflected by a change in the total lymphocyte count. The number of lymphocytes per mm^3 , however, was not significantly different among the patients tested (Table 1), concurring with the observations of Knobloch *et al.*⁸. Thus, the inversion we observed is presumably due to a loss of T cells and simultaneous increase in B cells during the first trimester, followed by a reversion of T and B cells to normal levels during the second trimester. To test this hypothesis, we are doing longitudinal studies of T and B cell levels in pregnant women. The initial blood test of seven women, 11–15 weeks pregnant, have already shown the expected inversion of T- and B-cell percentages in each patient.

Table 1 Percentages of T and B lymphocytes in pregnant and non-pregnant women

Donor	WBC per mm ³	Lymphocytes per mm ³	Weeks of gestation	% B	% T
Jen	6,400	1,792	NP	28	67
Deb	5,800	1,566	NP	24	71
Any	7,100	1,917	NP	28	70
Mar	6,300	ND	NP	29	64
Bur	7,300	1,606	7	62	30
Hor	4,300	1,247	7	69	24
And	8,200	ND	7.5	62	43
Wil	ND	ND	8	66	22
Bar	5,100	1,326	10	62	31
Hol	ND	ND	12	72	26
Joh	11,200	ND	15	62	28
Flo	7,900	2,607	17	57	31
Wea	6,200	ND	18	45	51
Key	ND	ND	19	48	49
Tho	15,000	ND	22	26	68
Und	ND	ND	22	29	63
Bow	ND	ND	23	26	69
Cam	ND	ND	23	28	68
Smi	5,800	1,160	25	31	72
Bjo	5,300	1,113	26	31	59
Par	5,100	2,244	34	27	68
Bil	ND	ND	35	38	62
Bai	4,000	ND	38	27	60
Can	ND	ND	40 (term)	30	66

NP, non-pregnant; ND, not done; WBC, white blood cells.

When percentages of T and B lymphocytes were plotted against weeks of pregnancy (Fig. 1), our data indicated a biphasic lymphocytic response which separates pregnancy into an early inversion phase and a stable normal phase. The inversion phase occurs during the first half of pregnancy with its peak effect between 7 and 15 weeks of gestation. This phase is characterised by a marked reduction in the percentage of T cells and a concomitant increase in the percentage of B cells. This alteration of normal T and/or B-cell levels during the inversion phase must be related to the implantation of the foetal allograft.

The stable normal phase of the biphasic lymphocytic response occurs during the latter half of pregnancy (week 22 to term) and is characterised by a return to essentially normal T and B cell levels. Since the re-establishment of normal numbers of T and B cells occurs at approximately 22 weeks, the foetal allograft may have already overcome any potential that might have existed for rejection by a T-cell-mediated reaction. Our observations may relate to reports indicating a deficiency in maternal immunological competence during pregnancy. T-cell deficiencies are suggested by the increased incidence of rubella⁹ and varicella¹⁰

infections as well as coccidioidomycosis¹¹ during pregnancy. *In vitro* evidence of altered maternal immunocompetence is based on maternal lymphocyte unresponsiveness to paternal but not unrelated histocompatibility antigens in the mixed lymphocyte reaction², and to a loss of maternal T-cell functions as shown by phytohaemagglutinin (PHA)-induced transformation¹. No significant decrease in PHA-induced transformation of maternal lymphocytes *in vitro* was, however, observed by Thiede¹² and Watkins¹³. Other evidence suggests that pregnancy plasma reversibly suppresses the response of maternal and normal lymphocytes in the mixed lymphocyte culture reaction¹⁴ as well as to PHA-induced transformation^{15,16}. In view of our results, a re-examination of maternal immunocompetence especially during the T and B-cell inversion phase, seems indicated. In addition, studies with purified T and B cells may be valuable in reassessing the functional immunological capabilities of pregnant women.

We suggest that the inversion of T and B-cell levels in early pregnancy may represent a physiological depletion of suppressor T cells (that is cells which are purported to be responsible for the regulation of some B-cell functions¹⁷) in response to the pregnancy which allows the number of B cells to increase during the first trimester. This increase in B-cell levels may assist in allograft acceptance through the production of blocking antibodies or factors similar to those suggested by the Hellström group for tumours¹⁸ and indeed reported to exist in pregnancy^{19,20}. Since the rise and fall of T and B-cell percentages seems to parallel the reported rise and fall of serum human chorionic gonadotrophin (HCG) levels²¹, and as Han²² has reported an inhibitory effect of HCG on the cell mediated immunity of guinea pigs *in vivo* and *in vitro*, it would be intriguing to attribute an immunoregulatory role to this hormone. Women receiving HCG fertility therapy are being tested to examine this possibility.

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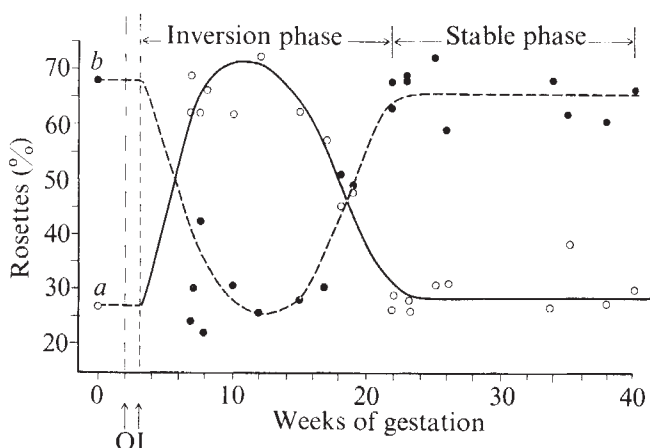
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Fig. 1 Levels of B(○) and T cells(●) were examined in 20 pregnant women who were in different weeks of gestation. Ovulation (O) was estimated to have occurred by 2 weeks and implantation (I) by week 3. Four women who were not pregnant were tested to determine the average control level of B (a) and T cells (b).



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Inhibition of mammalian cytotoxic cells by phosphatidylcholine and its analogue

MUCH interest has focused recently on the elucidation of cytotoxic mechanisms in cellular immunology. In addition to complement-mediated cytolysis, two major cell-mediated reactions have been realised from *in vitro* studies: the cytotoxic T-cell^{1,2} and K-cell^{3,4} systems. The former is characterised by the killing of target cells by cytotoxic T cells taken from donors previously sensitised to target cells, whereas in the latter, target cells are destroyed by normal or unsensitised effectors (K cells), provided IgG antibody to the target cell is present. The exact nature of the K cell is still a matter of controversy^{5,6}. Although a voluminous literature exists, little is known about the actual mechanism(s) of action of cytotoxic lymphoid cells. We describe here experiments which implicate phospholipase A activity as an early step of the kill mechanism in K-cell cytotoxicity.

The initial hypothesis for which these experiments were designed is as follows: it can be envisaged that some enzyme on the effector cell surface becomes activated by specific effector cell-target cell contact and alters the molecular architecture of target membrane to result in its lysis. The enzyme may be bound on the effector cell surface or actually be an integral plasma membrane protein. Such a mechanism could explain how the effector cell escapes being killed in the process, for it does remain functional, in both the K-cell⁷ and T-cell⁸ systems. One likely candidate enzyme would be phospholipase A (EC 3.1.1.4), for its activity leads to the conversion of lecithin (phosphatidylcholine) to lysolecithin+fatty acid, the former product being a very potent lytic agent. Using the rationale that such enzyme activity would be inhibited by various agents which should inhibit this activity, a synthetic inhibitor of phospholipase A (Rosenthal's inhibitor) and a normal phospholipid substrate for this enzyme were tested for possible inhibitory effects in a murine K-cell cytotoxic system.

The assay system used is a modification from Perlmann and Perlmann⁷, consisting of spleen effector cells from normal BALB/c female mice+adult chicken erythrocytes (⁵¹Cr-labelled)+BALB/c hyperimmune antiserum against adult chicken erythrocytes.

Rosenthal's inhibitor⁹, or DL-2,3-distearoyloxypropyl-(dimethyl)-(2-hydroxyethyl)-ammonium acetate (Calbiochem), a synthetic analogue of lecithin and inhibitor of phospholipase A, was examined for inhibitory capacity in this assay system. Figure 1 shows the dose-response effect

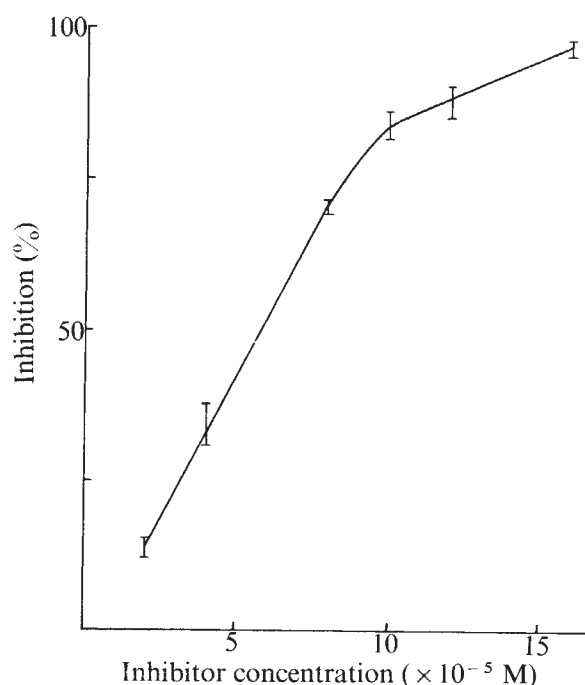


Fig. 1 Dose-response relationship of Rosenthal's inhibitor in murine K-cell cytotoxicity. Cytotoxic cultures (in triplicate) consisted of 2.5×10^6 BALB/c spleen cells + 10^5 chicken erythrocytes, ⁵¹Cr-labelled, $\pm$ BALB/c anti-chicken erythrocyte antiserum (1/1,500 final concentration) in a total volume of 1.5 ml medium 199 containing FCS (5%), penicillin (100 U ml⁻¹) and streptomycin (100 μ g ml⁻¹). Incubation was performed in a 10% CO₂ high humidity incubator at 37 °C. Rosenthal's inhibitor was added at various concentrations, and the cultures were incubated overnight (19 h). Results are expressed in terms of percentage inhibition of cytotoxicity:

$$1 - \frac{\% \text{ specific release in experimental}}{\% \text{ specific release in control}} \times 100$$

The percentage specific release is defined as the percentage release in cultures containing anti-target cell antiserum minus the percentage release in cultures containing normal mouse serum.

of this analogue on the cytotoxic reaction; a 50% inhibition is noted at 6×10^{-5} M. At an inhibitor concentration that results in more than 90% inhibition, no greater than a 10% reduction in spleen cell viability was noted, as judged by Trypan blue exclusion. Thus the inhibition effect cannot be due to simple nonspecific toxicity. Interference with the

Table 1 Reversibility of PC inhibition

Experiment	Prewash conditions	% Specific release at			
		1.5 h	3.0 h	4.0 h	4.5 h
1	Hanks'	5.8 (0.5)*	26.5 (0.9)	31.3 (2.2)	—
	PC 100	0.3 (0.7)	21.5 (0.5)	27.1 (0.5)	—
2	Hanks'	7.3 (1.3)	21.9 (0.5)	26.2 (2.3)	—
	PC 100	3.5 (0.8)	16.5 (1.4)	20.9 (2.0)	—
3†	Hanks'	8.1 (0.7)	29.5 (1.8)	—	38.3 (3.6)
	PC 100	3.7 (0.5)	23.7 (0.7)	—	33.4 (0.8)
4	Hanks'	6.1 (0.5)	28.1 (1.3)	33.1 (1.8)	—
	PC 200	1.3 (0.4)	9.8 (0.6)	13.6 (0.4)	—
5	Hanks'	5.4 (0.6)	37.2 (1.9)	—	42.3 (0.6)
	PC 200	2.1 (0.8)	13.5 (1.5)	—	17.4 (1.5)
6†	Hanks'	5.3 (0.7)	37.4 (0.1)	48.2 (0.4)	—
	PC 200	2.1 (0.4)	30.6 (1.5)	41.6 (0.1)	—
7†	Hanks'	12.1 (0.6)	55.8 (0.9)	65.6 (0.7)	—
	PC 200	4.1 (0.4)	50.1 (0.1)	60.8 (3.0)	—

Cytotoxic mass cultures, in the presence or absence of PC at 100 (PC 100) or 200 (PC 200) μ g ml⁻¹ final concentration, were incubated at 37 °C for 90 min; aliquots were then removed to determine percentage specific release and the remaining cells were washed free of PC by centrifugation through a 20% (w/v) bovine serum albumin solution prepared in Hanks' BSS. The washed cells were resuspended in fresh medium and reincubated for further 90, 150 or 180 min to assess the degree of reversibility.

*Standard deviation.

†Experiment done in presence of 5% FCS.

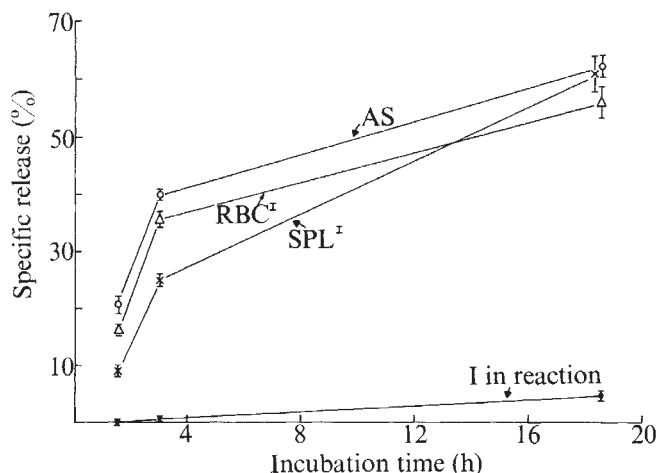


Fig. 2 Rosenthal's inhibitor pretreatment of effector or antibody-coated target cells. Spleen effectors or antibody-coated targets were pretreated with Rosenthal's inhibitor (8×10^{-8} M) for 30 min at 37°C , washed in medium 199 and tested for cytotoxicity after various times. $\circ$, Positive control, no inhibitor; Δ , antibody-coated target cells pretreated with inhibitor; $\times$, spleen effector cells pretreated with inhibitor; $\bullet$, inhibitor present during cytotoxic reaction.

recognition phase of the reaction (K cell Fc-receptor attachment to the Fc portion of target-bound antibody) is unlikely as EA rosette formation (mouse spleen + rabbit anti-SRBC-coated SRBCs (sheep red blood cells)) is unaffected by Rosenthal's inhibitor. As one would expect of a competitive inhibitor, pretreatment of effector or target cells was not very effective for determining the cell type affected (Fig. 2); however, the reduced cytotoxicity at early times for the pretreated spleen cells (56% at 1.5 h; 37% at 3.0 h) suggests that the inhibitor affects predominantly the effector cell population. Reduction is not attributable to diminished numbers of effector cells, for both control and pretreated spleen cells were adjusted to the same number of viable cells.

Phosphatidylcholine (PC) was next examined for possible inhibition of the cytotoxic reaction. Addition of distearoyl-PC (Calbiochem) to the cytotoxic cultures caused inhibition—50% at $37 \mu\text{g ml}^{-1}$ or 4.6×10^{-5} M (Fig. 3). As for Rosenthal's inhibitor, PC had no effect on EA rosette formation. The inhibition by PC at $100 \mu\text{g ml}^{-1}$ was completely reversible, with no time lag, following removal of the phospholipid by centrifugation and resuspension in fresh medium; at $200 \mu\text{g ml}^{-1}$, however, the reversal was complete only in the presence of serum in the cultures (Table 1) while in the other experiments, presence or absence of serum had no effect. The site of PC inhibition seems to be on the effector cell, for spleen cell pretreatment with PC results in diminished effector cell function, provided pretreatment is carried out in the absence of foetal calf serum.

The protective effects of Rosenthal's inhibitor and PC against target cell injury strongly suggest that phospholipid alteration by phospholipase A is the basis for the loss of membrane integrity in these cytotoxic reactions. The fact that neither agent affects EA rosetting efficiency argues against interference with cell-cell contact as the mechanism whereby these substances inhibit the cytotoxic reaction. The most probable manner by which PC inhibits the cytotoxic reaction is as follows: exogenous PC may act as a substrate for phospholipase A, thus preventing the enzyme from attacking endogenous target cell membrane phospholipids. The lysophosphatidylcholine (LPC) thus generated by the enzyme activity would not subsequently lyse the target cell since excess PC and effector cell surface would be expected to bind preferentially the LPC generated, because of their more intimate association with the proposed

enzyme sites on the effector cell. The fact that reversibility of PC inhibition at $200 \mu\text{g ml}^{-1}$ is only partial in the absence of serum strongly indicates that LPC is generated and interferes with effector cell function. Effector integrity is not affected when serum is present in the reaction mixture, and the reason for the protective effect of serum probably lies in the fact that serum albumin binds LPC and renders it non-lytic⁹.

It is interesting that the observed PC inhibition parallels that of EDTA¹⁰ in that both seem to act at a late stage in the cytolytic reaction (since there is no time lag in chromium release when inhibitor is removed). As most phospholipase A enzymes are calcium dependent¹¹ and thus inhibited by EDTA, it is tempting to speculate that both PC and EDTA act at the same point in the cytotoxic sequence, namely at that point requiring phospholipase A activity.

Experiments are in progress to assess the feasibility of obtaining direct chemical evidence for the generation of

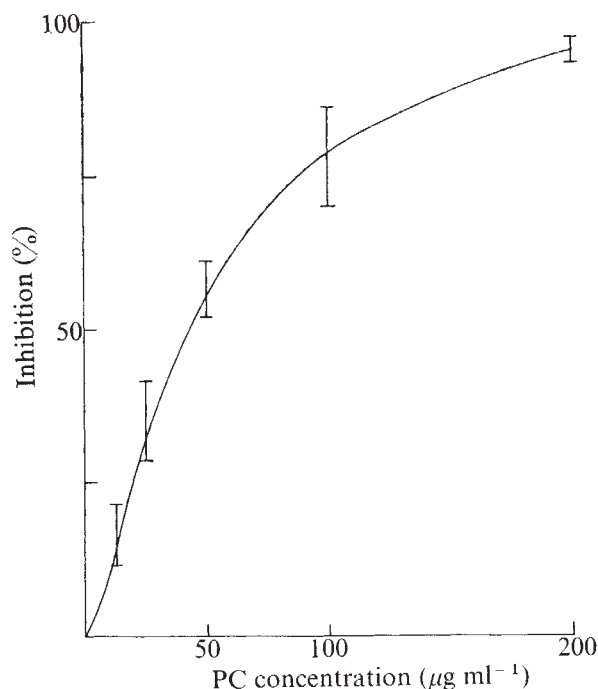


Fig. 3 PC inhibition of murine K-cell cytotoxicity. Same conditions as in Fig. 1, except that incubation was for 3 h and reactions were carried out in the absence of foetal calf serum. To obtain an aqueous dispersion, PC was sonicated in Hanks' BSS for 5 min, using a Biosonik IV sonicator (low setting 50). The results are expressed as the means $\pm$ s.d. of three experiments.

LPC in the target cell membranes during the cytotoxic reaction.

Any hypothesis implicating phospholipase A in the kill phase mechanism must not overlook the fact that most such enzymes have been found to be incapable of attacking the phospholipids of intact plasma membranes¹²; only in the presence of membrane-perturbing agents such as hypotonic milieu¹³, detergents¹⁴ or DLF¹⁵ (direct lytic factor, a small basic peptide found in snake venoms) do such enzymes catalyse the conversion of intact membrane phospholipids into their lyso-derivatives. Consequently, a two-step reaction can be postulated: a preparatory step in which the membrane is perturbed, and a second step in which phospholipase A is active. Efforts are under way to assess the possible nature of such a preparatory step.

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Immunological restoration of both thymectomised and athymic nude mice by a thymus factor

AN endocrine function for the thymus has been suggested by experiments using both thymic tissue enclosed in diffusion chambers and cell-free thymus extracts to restore the immunological functions in neonatally thymectomised animals¹⁻⁴. Several investigators have prepared a variety of thymic humoral factors (thymic hormones) capable of restoring different immunological functions⁵, but so far they have had little success in endowing congenitally athymic nude mice with T cells and T-dependent functions⁶, although thymus grafts work very well⁷. This has led to the suggestion that thymic humoral factors may work by promoting the maturation of lymphocytes which have already passed through the thymus. The study presented here, however, shows evidence that a thymus factor substitutes for the thymus.

The first step was to verify *in vivo* the capacity of a thymus factor (TF) to reject tumour allografts in thymectomised and athymic nude mice. The assay method of purification of thymic hormone has presented many inherent difficulties especially *in vitro*: for example, it has been reported that a great variety of mitogenic and non-mitogenic substances, including those which raise the intracellular concentration of cyclic AMP, can induce *in vitro* the differentiation of stem cells to θ -positive lymphocytes and immunocompetent cells⁸⁻¹⁰.

The TF was prepared from calf thymus using a modification of the method described by Goldstein *et al.*¹¹. The same

procedure, up to and including the heat treatment, was followed. Then saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 6.8) was added to a concentration of 50% saturation. The precipitate was collected by centrifugation and dissolved in saline. Free $(\text{NH}_4)_2\text{SO}_4$ was removed from this solution on a column of Sephadex G-25. The protein fractions were combined and diluted with saline to a final protein concentration of either 1 or 2 mg ml⁻¹. The same fractionation procedure from calf spleen was performed as a control.

Fibrosarcoma cells, induced originally by a subcutaneous injection of 3-methylcholanthrene into a C3H/He (H-2^k) mouse, were used as tumour grafts. A/J (H-2^d) mice were used as recipients of tumour allografts in standard laboratory conditions. In the first experiment, A/J mice were thymectomised within 24 h after birth. From the following day, the mice were subcutaneously injected five times a week with 0.2 mg of either TF, or, as controls, spleen extract (SE) or bovine serum albumin (BSA). On the day after the 10th injection, 2×10^6 fibrosarcoma cells were inoculated subcutaneously into the backs of the mice. After a further 20 injections with TF, SE, or BSA, the tumour incidence was noted in each group. In the 24 mice injected with TF, no tumours were found, whereas, of the 20 mice injected with BSA, 14 developed tumours and the remaining 6 died of 'wasting disease' before tumour development. Of the 22 mice injected with SE, 13 developed tumours and the remaining 9 died of 'wasting disease'. In the second experiment, A/J mice were thymectomised 3-5 d after birth to avoid death from wasting disease following neonatal thymectomy. The experimental schedule followed after the thymectomy is shown in Table 1. Of the 34 mice injected with TF, 31 rejected the tumours and survived the 90-d period. Of the mice injected with BSA or SE no tumours were rejected, and after the 90-d period there were none surviving (Table 1). In the third experiment, athymic nude mice (backcrossed on to BALB/c (H-2^d) mice) were used. Nude mice obtained from the Central Institute for Experimental Animals, Tokyo, were raised in specific pathogen-free conditions. Male athymic nude mice (4 weeks old) were subcutaneously injected every day with 0.5 mg of either TF or SE. In the case of athymic nude mice, no rejection took place when TF was injected after inoculation of the tumour cells. No tumours were, however, detected when 2×10^6 fibrosarcoma cells were inoculated into 5 athymic nude mice which had been pretreated 60 times with TF. Histological findings showed a remarkable accumulation of lymphocytes in the location of the tumours undergoing rejection. Moreover, to verify *in vitro* the cytotoxic effects on the tumour cells, a microcytotoxicity assay was used¹². Spleen cells from both thymectomised and athymic nude mice which had rejected tumours showed evidence of contact with the tumour cells followed by destruction of the latter. Nonetheless, incubation with 1:4 dilution (final dilution 1:12) of the anti-Thy 1.2 (θ -C3H) serum and complement completely abolished the cytotoxic activity of their spleen cells (Table 2). In the treatment of

Table 1 The effect of injection of a thymus factor into thymectomised A/J mice on the rejection of allogeneic tumour grafts of greatest diameter of between 5 and 10 mm

Injection	No. of mice rejecting tumours	No. of mice dead within 30 d	No. of mice surviving 90 d
Bovine serum albumine (BSA)	0/24 (0%)	8/24 (33%)	0/24 (0%)
Thymus factor (TF)	31/34 (91%)	3/34 (9%)*	31/34 (91%)
Spleen extract (SE)	0/24 (0%)	6/24 (25%)	0/24 (0%)

A/J (H-2^d) mice were thymectomised 3-5 d after birth. Two days later, 2×10^6 fibrosarcoma cells of C3H/He(H-2^k) origin were inoculated subcutaneously into the backs of the mice. After a period of 10-20 d, when the tumour size reached 5-10 mm at the greatest diameter, the mice were subcutaneously injected five times a week with 0.5 mg of BSA, TF, or SE. After 20 injections, the number of mice rejecting tumours was noted.

*Statistically significant difference from the control (BSA) values, $P < 0.05$.

Table 2 *In vitro* cytotoxic effects of spleen cells and sera from thymectomised A/J mice and athymic nude mice subjected to long term injections of either thymus factor (TF) or spleen extract (SE), on allogeneic tumour cells

Mouse†	Injection	Tumour rejection	% Cytotoxicity (mean ± s.e.m.)					
			Cell-mediated cytotoxicity*			Antibody-mediated cytotoxicity		
			(at 48 h)			(at 12 h)		
			Treatment with anti-θ serum + C					
			Anti-θ serum dilution		Mouse serum dilution			
				1:4	1:16	1:1	1:4	
Tx A/J (5)	SE (0.5 mg × 20)	no	6.3 ± 2.2	1.2 ± 0.5	ND	8.5 ± 3.2	ND	
Tx A/J (5)	TF (0.5 mg × 20)	yes	89.4 ± 8.3	1.8 ± 0.3	22.3 ± 6.1	19.5 ± 4.8	8.6 ± 3.2	
nu/nu (3)	SE (0.5 mg × 60)	no	1.8 ± 0.4	ND‡	ND	1.3 ± 0.4	ND	
nu/nu (3)	TF (0.5 mg × 60)	yes	62.5 ± 6.3	2.1 ± 0.6	24.7 ± 5.3	16.5 ± 6.8	9.0 ± 4.1	

A microcytotoxicity assay was performed by using spleen cells and sera from both thymectomised and athymic nude mice which had rejected tumour allografts following TF-treatment, and also from those which had shown no tumour rejection.

*Effector-target cell ratio = 50:1.

†Numbers in brackets indicate the number of mice in the group.

‡ND, not done.

their spleen cells with complement alone, anti-Thy 1.2 serum alone, and normal AKR mouse serum plus complement, none of them abrogated the cytotoxic activity of their spleen cells. In comparison with their spleen cells, sera from both thymectomised and athymic nude mice which had rejected tumour allografts following TF-treatment showed only a slight cytotoxicity. In the experiments, cell-mediated cytotoxicity had a far more important role than either cell-mediated cytotoxicity which was antibody-dependent (data not shown) or antibody-mediated cytotoxicity. The results show that a thymus factor induces, in

both thymectomised and athymic nude mice, 'killer T cells' which have a major role in the rejection of tumour allografts.

The next step was to consider whether helper T cells could be induced by TF in athymic nude mice. Nude mice which had been injected 60 times with 0.5 mg of TF or SE were used. On the third day after the last injection, the mice thus treated, and untreated *nu/+* control mice, were immunised intraperitoneally with 4×10^8 sheep red blood cells (SRBCs). Five days later the number of haemolytic plaque-forming cells (PFCs) in their spleens was determined using Cunningham's¹³ technique. The direct splenic PFC response of the TF-treated *nu/nu* mice was increased to $9,526 \pm 2,326$ and the indirect PFC response to $92,160 \pm 4,366$, both of which were near the normal level of *nu/+* mice. The response of the SE-treated *nu/nu* mice, on the other hand, did not show a statistically significant difference from that of the untreated *nu/nu* mice (Table 3).

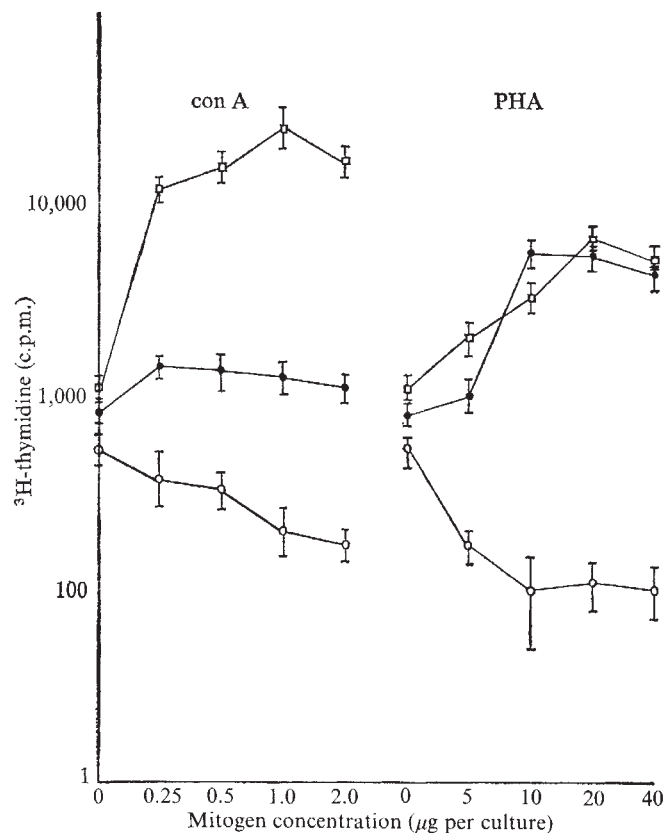


Fig. 1 Effect of a thymus factor (TF) on the responsiveness to PHA and con A of *nu/nu* spleen cells. Two four-week-old male *nu/nu* mice were subcutaneously injected every day with 0.5 mg of TF for 60 d. Three days later, the mitogenic reactivity of their pooled spleen cells was determined by measuring the incorporation of ³H-thymidine into DNA. ○, Untreated *nu/nu* mice; ●, TF treated *nu/nu* mice; □, BALB/c mice. Vertical bars and points show the mean ± s.e.m.

Table 3 The effect of a thymus factor on the number of direct and indirect PFCs in the spleens of *nu/nu* mice

Mouse	Injection	PFCs per spleen after 5 d*	
		Direct	Indirect
<i>nu/nu</i>	No	176 ± 152	1,123 ± 221
<i>nu/nu</i>	TF (0.5 mg × 60)	9,526 ± 2,326	92,160 ± 4,366
<i>nu/nu</i>	SE (0.5 mg × 60)	246 ± 110	1,236 ± 312
<i>nu/+</i>	No	19,932 ± 8,421	108,415 ± 21,513

Four-week-old male *nu/nu* mice were subcutaneously injected every day with 0.5 mg of TF or SE for 60 d. On the third day after the last injection, all three mice in each of the four groups were immunised intraperitoneally with 4×10^8 SRBCs. After 5 d, the number of PFCs in the spleens was determined.

* Means ± s.e.m.

Histological findings in the lymph nodes and spleens from the TF-treated *nu/nu* mice showed an accumulation of lymphocytes in T-cell dependent areas. Moreover, to verify T-cell induction in the TF-treated *nu/nu* mice, the cytotoxicity test using anti-Thy 1.2 serum and complement was performed as described by Boyse *et al.*¹⁴. Spleen cells and lymph node cells from the SE-treated *nu/nu* mice showed cytotoxic indices of 0.5 and 0.7%, respectively, whereas spleen cells and lymph node cells from the TF-treated *nu/nu* mice showed indices of 23 and 39%, respectively. These findings confirm the induction of T cells by TF in *nu/nu* mice.

The next experiment was to determine the capacity of TF to restore lymphocyte responsiveness to the T-cell

mitogens phytohaemagglutinin (PHA) and concanavalin A (con A) in TF-treated *nu/nu* mice. We used pooled spleen cells from two *nu/nu* mice which had received the same treatment with TF as in the experiment already described. Age-matched and sex-matched controls consisted of untreated *nu/nu* and ordinal BALB/c mice. The mitogenic reactivity of each spleen cell was determined by measuring the incorporation of ^3H -thymidine into DNA. We then set up 5×10^5 spleen cells in 0.2 ml of culture medium (RPMI 1640 medium containing 10% foetal calf serum) in wells of microtrays (Limbro) and cultured them in triplicate for 72 h. We added 10 μl of culture medium containing the concentrations of PHA-P (Difco) and con A (Calbiochem.) indicated in Fig. 1. Twenty-four hours before collection, 1 μCi of ^3H -thymidine was introduced into each well. Labelled material was measured using liquid scintillation counting. Spleen cells from the untreated *nu/nu* mice did not respond to either of the mitogens (Fig. 1). In contrast, spleen cells from the *nu/nu* mice treated with TF showed a remarkable response to PHA. The level was almost the same as that shown by the BALB/c mice. In contrast with the response to PHA, the *nu/nu* spleen cells treated with TF did not show a response to con A of a level comparable with that of the BALB/c mice, although they did show a statistically significant difference from that of the untreated *nu/nu* spleen cells ($P < 0.001$). It is difficult to explain this dissociation of mitogenic responses to PHA and con A, but T cells induced by this thymus factor may correspond to the TH-2b subclass described by Dyminski *et al.*¹⁵, which responds significantly to PHA and SRBC but poorly to con A and mixed lymphocyte culture (MLC). Van Bekkum *et al.*⁷ have reported that it is impossible to restore the PHA responsiveness of athymic nude spleen cells after long term treatment *in vivo* with thymus extracts (thymosin and THF). Our study, however, has demonstrated that the thymus factor is capable of restoring PHA responsiveness and the other T-dependent functions in athymic nude mice. This thymus factor is still crude and may turn out to be a mixture of a few active components of the thymus; it is also possible that more than one component of thymus factor are needed for thymic hormone to substitute for the thymus.

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Calcium-dependent stimulation of platelet aggregation by PGE₂

THERE is considerable interest in the effects of prostaglandins on blood platelets, partly because the synthesis of prostaglandins by stimulated platelets is important in haemostasis, and partly because platelets can be used as models to investigate the actions of prostaglandins (PGs) at a cellular level¹. The effects of different PGs on platelets are diverse: PGE₁ is a potent inhibitor of platelet aggregation² with a K_i value around 30 nM and PGD₂ is an even more effective inhibitor of aggregation of human platelets, but not those of some other species^{3,4}. PGE₂ inhibits platelet aggregation at high concentrations ($> 1 \times 10^{-6}$ M), and there is some controversy as to whether lower concentrations enhance aggregation⁵. When human platelets are stimulated by collagen they synthesise PGE₂ from arachidonic acid⁶ and the cyclic endoperoxide intermediates in the biosynthesis of PGE₂ exert a direct aggregating effect^{7,8}. Synthetic derivatives of PGE₂ can also induce the aggregation of human platelets⁹, but PGE₂ itself has never been found to induce aggregation directly.

Previous studies of the effects of PGs on platelet aggregation used platelets suspended in citrated plasma (with most of the calcium chelated) or in calcium-free artificial media. The effects of PGE₁ on several biological systems are influenced by the ionised calcium concentration^{10,11} and platelet aggregation is also calcium dependent to a variable extent in different animal species¹². We have investigated the effects of PGE₁, PGE₂ and

Table 1 Inhibition of collagen-induced platelet aggregation by prostaglandins

Species	Anticoagulant	Concentration ($\times 10^{-6}$ M) inhibiting aggregation by 50%		
		PGE ₁	PGD ₂	PGE ₂
Man	Citrate	0.015	0.008	6.0
	Heparin	0.054	0.015	22.0
Rat	Citrate	0.06	> 200	75
	Heparin	0.09	> 200	135
Pig	Citrate	0.27	1.4	67
	Heparin	0.12	0.15	*

*Directly induces aggregation.

Prostaglandins or saline were preincubated for 1 min at 37 °C in PRP before the addition of collagen. In each experiment, the dose of collagen producing a half-maximal rate of aggregation was used as the aggregation stimulus. Results are expressed as mean IC₅₀ values ($\times 10^{-6}$ M) of three determinations.

PGD₂ on platelet aggregation in heparinised and citrated platelet-rich plasma (PRP) from man, pig and rat, and have found that PGE₂ reproducibly enhances aggregation in heparin PRP (but not in citrate PRP) from man and rat, and induces aggregation directly in pig heparin PRP.

Human blood was obtained by antecubital venepuncture from volunteers who had ingested no aspirin within the previous 10 d. Pig blood was obtained from a cannula inserted into the carotid artery of animals under Nembutal anaesthesia, and rat blood was obtained by venepuncture from the inferior vena cava of animals under light ether anaesthesia. The preparation of PRP and the measurement of platelet aggregation induced by collagen have been described in detail^{13,14}. Drugs or saline were preincubated in PRP for 1 min at 37 °C before the addition of collagen. To compensate for minor variations in the platelet aggregation response during the course of the experiments, controls were performed before and after each test determination. PGE₁, PGE₂ and PGD₂ were dissolved in 95% ethanol and converted to the sodium salt by addition of 9 volumes of sodium carbonate (1.9×10^{-3} M). Stock solutions (3×10^{-3} M) were stored under nitrogen at -20 °C. Fresh samples were thawed before each experiment, and diluted with iso-osmotic saline.

The potencies of PGE₁, PGE₂ and PGD₂ as inhibitors of platelet aggregation in the three species tested are shown in Table 1. PGE₁ was the only PG which inhibited aggregation in both heparin PRP and citrate PRP in all three species. It was most potent in man, slightly less so in the rat, and least potent in the pig. The IC₅₀ values (concentration producing 50% inhibition of aggregation) ranged from 0.015×10^{-6} M in human citrate PRP to 0.27×10^{-6} M in pig citrate PRP. PGD₂ was more potent than PGE₁ in man ($IC_{50} = 0.008 \times 10^{-6}$ M in citrate PRP), less potent than PGE₁ in the pig, and was inactive in the rat. PGE₂ was much less potent than PGE₁ in all species, with IC₅₀ values in citrate PRP of 6×10^{-6} M in man, 67×10^{-6} M in the pig, and 75×10^{-6} M in the rat. In man and rat, all three PGs were more potent inhibitors in citrate PRP than in heparin PRP. In the pig, the reverse was true, and PGE₂ was unique in causing aggregation directly in pig heparin PRP. PGE₂ never induced aggregation in human and rat PRP, but with concentrations in the range 0.3×10^{-6} – 30×10^{-6} M in the rat and 0.3×10^{-6} – 3.0×10^{-6} M in man, collagen-induced aggregation was reproducibly enhanced in heparin PRP though not in citrate PRP. Dose-response curves for the enhancement and inhibition of aggregation by PGE₂, and for inhibition of aggregation by PGD₂ and PGE₁ are shown in Fig. 1.

When PGE₂ was added to pig heparin PRP at concentrations $\geq 0.5 \times 10^{-6}$ M there was an immediate aggregation response which was not preceded by any detectable change in platelet

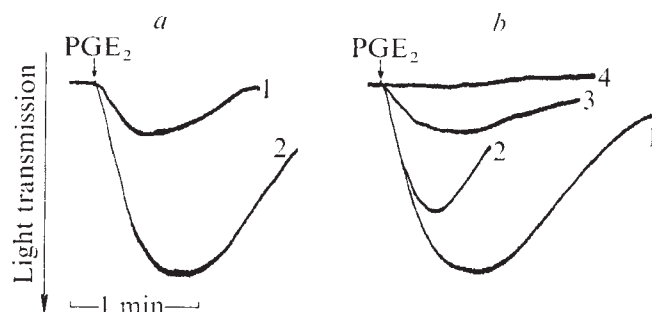
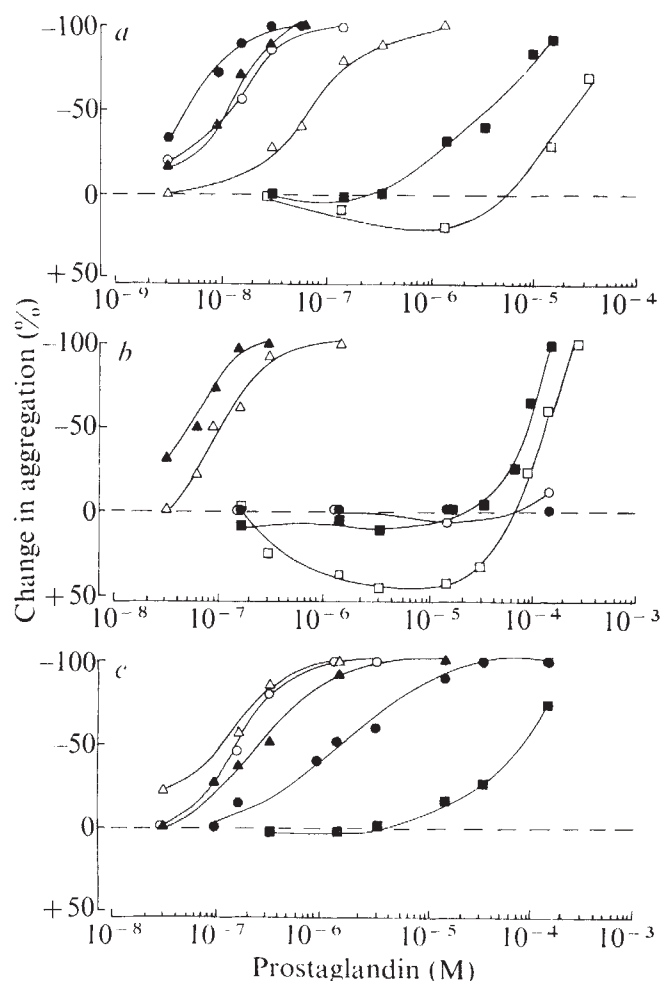


Fig. 2 *a*, Platelet aggregation responses induced by PGE₂ in pig heparin PRP. 1, 1.5×10^{-6} M PGE₂; 2, 1.5×10^{-5} M PGE₂. *b*, Inhibition of aggregation induced by PGE₂ in pig heparin PRP. PGE₂ (1.5×10^{-5} M) was added to samples preincubated for 1 min with: 1, saline; 2, EDTA (3×10^{-3} M); 3, PGD₂ (1.5×10^{-6} M); 4, PGE₁ (1.5×10^{-7} M).

shape. The aggregation response was dose dependent, being small and reversible at concentrations $\sim 0.5 \times 10^{-6}$ – 5.0×10^{-6} M, and larger and more slowly reversible at higher concentrations (Fig. 2*a*). Aggregation induced by PGE₂ in pig heparin PRP was inhibited by EDTA (3×10^{-3} M), trisodium citrate (12.5×10^{-3} M), PGE₁ and PGD₂ (Fig. 2*b*); the aggregating effect of a given concentration of PGE₂ could be abolished by a concentration of PGE₁ 100 times lower. Platelet aggregation induced by PGE₂ in pig heparin PRP did not depend on the presence of heparin; similar aggregation responses were obtained when PGE₂ was added to partially recalcified pig citrate PRP.

Vigdahl *et al.*¹⁰ showed that ionised calcium antagonised the inhibitory effect of PGE₁ on ADP-induced aggregation of human platelets. Our results suggest that this antagonism is not limited to PGE₁, nor to platelet aggregation induced by ADP, since inhibition of collagen-induced aggregation of human and rat platelets by PGE₁, PGE₂ and PGD₂ was less in heparin PRP than in citrate PRP. Pig platelets were unusual in that PGE₁ and PGD₂ were more potent inhibitors in heparin PRP than in citrate PRP. The reason for this discrepancy is unknown, but several observations have demonstrated that the function of calcium in pig platelet aggregation is different from that in other species^{12,15,16}. Indeed, the modification of PG effects by calcium is most marked in the case of PGE₂ in pig platelets: PGE₂ inhibited aggregation in citrate PRP, but induced aggregation directly in heparin PRP or recalcified citrate PRP. This aggregation induced by PGE₂ is the first demonstration of a direct platelet-aggregating effect induced by a stable, naturally-occurring prostaglandin. PGE₂ has been shown to potentiate platelet aggregation induced by endoperoxide intermediates in the PGE₂ biosynthetic pathway, and such aggregation (measured in human citrate PRP) was also enhanced by the addition of ionised calcium¹⁷. Our observation that collagen-induced aggregation of human and rat platelets in heparin PRP can be enhanced by subinhibitory concentrations of PGE₂ supports the concept that PGE₂ can exert a stimulatory effect on platelets through a calcium-dependent mechanism. It seems likely that the stimulatory effect of PGE₂ on human and rat platelets, and its direct aggregating effect on pig platelets, could be mediated by an influx of extracellular calcium; platelets can be stimulated in this way by the calcium ionophore A23187 (ref. 18) and PGE₁ can stimulate calcium influx into other tissues^{11,19}. It is generally accepted that inhibition of platelet aggregation by PGs is mediated by stimulation of adenylate cyclase^{5,20}, but 10^{-5} M PGE₂ is required to stimulate human platelet adenylate cyclase, and collagen-induced aggregation of human platelets in heparin PRP is stimulated by PGE₂ at concentrations below 10^{-6} M. Similarly, the minimal effective concentration of PGE₂ which induces aggregation in pig heparin PRP is about 10^{-6} M, and the minimal inhibitory concentration in pig citrate PRP is around 10^{-5} M. Further studies are necessary to determine whether PGE₂ can, in fact,

Fig. 1 Dose-response curves for the modification of collagen-induced aggregation by PGE₁ (▲, △), PGE₂ (■, □) and PGD₂ (●, ○) in citrate PRP (▲, ■, ●) and heparin PRP (△, □, ○) of man, pig and rat. The aggregation stimulus in each case was the concentration of collagen producing a half-maximal rate of aggregation. Results (mean values, $n = 3$) are expressed as percentage change in the rate of aggregation. *a* Human; *b*, rat; *c*, pig.



stimulate the influx of calcium into platelets at concentrations lower than those which stimulate adenylate cyclase. Investigations of the mechanisms by which PGs exert their various biological effects are facilitated by the use of a homogeneous population of cells on which responses to different PGs can be readily determined. Since pig platelets contain receptors for PGE₁, PGE₂ and PGD₂, and both stimulatory and inhibitory effects can be obtained, the pig platelet could prove a valuable model for studying the interactions of calcium, prostaglandins and their cellular receptors.

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Human breast cancer responsive to androgen in long term tissue culture

ALTHOUGH a significant role has been established for oestrogen in the growth of some animal^{1,2} and human^{3,4} mammary carcinoma, evidence for androgen dependency of human breast cancer has remained equivocal. Although the existence of a mouse mammary carcinoma which shows modest androgen responsiveness⁵ has supported the notion that some human breast cancer might also be dependent on androgens, no direct proof has been available. We have described our work characterising the oestrogen responsiveness of MCF-7, a human breast cancer cell line maintained in tissue culture for over 3 yr (ref. 6). This cell line was shown to contain oestrogen receptor, to be killed by antioestrogens, and stimulated by physiological concentrations of oestradiol. We now describe

further investigations of this cell line which reveal that in addition to oestrogen responsiveness, this cell line shows threefold enhancement of precursor incorporation into macromolecules by androgens using serum-free conditions which preclude stimulatory effects of other trophic hormones. Furthermore, this stimulation by androgens seems to be mediated by interaction with a cytoplasmic androgen receptor protein which is clearly differentiable from the oestrogen receptor also found in these cells. Aside from defining an interesting new system in which the action of androgen can be studied in a human cell line in tissue culture, the present study provides unequivocal evidence for the androgen responsiveness of some human breast cancer at least *in vitro*.

Table 1 shows the effects of various steroids on incorporation of radiolabelled thymidine and leucine into acid precipitable material. As indicated, 5- α -dihydrotestosterone stimulates leucine and thymidine incorporation three- to fourfold above control levels. Testosterone also stimulates both thymidine and leucine incorporation though to a lesser degree than 5- α -dihydrotestosterone. At similar concentrations 5- β -dihydrotestosterone was inactive in stimulating macromolecular synthesis. R2956 (17- β -hydroxy-2,2,17- α -trimethyl-estra-4,9,11-trien-3-one), an antiandrogen provided by Dr J. P. Raynaud (Roussel, France)⁷, at a concentration of 10⁻⁶ M strongly inhibits thymidine incorporation. If the cells are left in R2956 longer than about 48 h, they begin to round up, detach from the surface of the dish and die. In experiments not shown, stimulation of precursor incorporation was generally not apparent before about 24 h of incubation, a lag period compatible with many steroid hormone effects⁸.

This stimulation of precursor incorporation was reflected by an overall stimulation of net protein synthesis and cell division as shown in Table 2. 5- α -Dihydrotestosterone (10⁻⁸ M) stimulated protein synthesis per dish, with differences apparent after 2 d in culture. After 8 d triplicate control dishes contained an average of 120,000 cells as compared with 172,000 cells in androgen stimulated dishes. Thus, 5- α -dihydrotestosterone (which unlike testosterone cannot be aromatised to oestrogen) is capable of stimulating cell proliferation significantly in this human breast cancer cell line *in vitro*. We ascribe the smaller increases in net protein synthesis and cell proliferation as compared with stimulation of precursor incorporation to the fact that the experiments were performed in serum-free conditions which markedly inhibit cell growth.

Based on many previous studies on the mechanism of action of androgens^{9,10} one would expect these cells to contain androgen receptor. Binding studies on cytoplasmic extracts using a dextran coated charcoal assay revealed high affinity limited capacity androgen binding molecules with affinity for both 5- α -dihydrotestosterone and testosterone. Scatchard analysis of the binding data revealed a class of receptors for 5- α -dihydrotestosterone of uniform affinity ($K_d = 7.5 \times 10^{-10}$ M, $r = 0.997$). When ³H-testosterone was used instead, K_d was 1.5×10^{-9} M ($r = 0.958$). The

Table 1 Effects of various steroids on incorporation of thymidine and leucine into acid precipitable material in MCF-7 human breast cancer

Steroid	³ H-thymidine incorporation (d.p.m. $\times 10^{-2}$ / μ g protein/h $\pm$ 1 s.d.)	¹⁴ C-leucine incorporation (d.p.m. $\times 10^{-1}$ / μ g protein/h $\pm$ 1 s.d.)
Control	10.2 $\pm$ 1	7 $\pm$ 1.1
10 ⁻⁶ M 5- α -dihydrotestosterone	41 $\pm$ 1	16.7 $\pm$ 0.9
10 ⁻⁶ M 5- β -dihydrotestosterone	9.6 $\pm$ 1.9	7.8 $\pm$ 0.6
10 ⁻⁶ M Testosterone	19.6 $\pm$ 1.9	10.5 $\pm$ 1.1
10 ⁻⁶ M R2956	5.6 $\pm$ 0.3	Not done

Cells growing in monolayer cultures as previously described⁶ were changed to serum-free Eagle's MEM. After 24 h the medium was replaced with fresh serum-free medium. Hormones were added as 1,000–10,000 times concentrates in ethanol. After 48 h the cells were pulsed with labelled compounds and incorporation into macromolecules measured as previously described. Purity of 5- α -dihydrotestosterone and testosterone were checked by thin-layer chromatography¹⁹.

Table 2 Effect of androgen on net protein synthesis in MCF-7 human breast cancer in tissue culture

	Time (d)				
	0	2	4	6	8
Control	25.8	30.8	35.5	38	43.1
+ 10^{-6} M 5- α -dihydrotestosterone	25.8	35.7	40.4	46.4	53.8

Cells growing in monolayer were suspended with trypsin-EDTA solution, and plated in triplicate in 60-mm Petri dishes at a density of about 10^5 cells per dish. After 24 h, regular growth medium was changed to Eagle's MEM without serum. Hormone was added to 1/2 the dishes and at various times cells were collected and washed and total cellular protein per dish was estimated after sonication in water by the Lowry technique²⁰. Values are in μ g protein per 50- μ l aliquot of homogenate.

approximately twofold lower dissociation constant for 5- α -dihydrotestosterone against testosterone suggests that the reduced compound may be the active androgen in these cells. The ability of these cells to convert testosterone to 5- α -dihydrotestosterone was examined directly by incubating cells in serum-free medium with 3 H-testosterone and examining the medium, total cell homogenate, and crude nuclear pellet prepared by differential centrifugation for formation of 3 H-5- α -dihydrotestosterone. Using methylene chloride to extract steroids, adding non-radioactive carrier steroids and running the organic phase on silica gel G plates using chloroform-methanol (98:2 v/v), we found counts migrating in the position of both 5- α -dihydrotestosterone and androstenediol in both the extracted media, cell homogenate and crude nuclear pellet. At 4 h, we estimated conversion to be 13.7%. This suggests that the cells do contain 5- α -reductase activity and that testosterone itself may be the precursor androgen in these cells *in vivo*. The cells rapidly metabolise 5- α -dihydrotestosterone to androstenediol and other more polar products. The description of these metabolic conversions will form the subject of a later communication but clearly the cells contain not only 5- α -reductase activity but 3-ketoreductase activity as well. In an experiment in which the number of binding sites for both compounds was quantified on a single cytosol preparation, specifically bound 5- α -dihydrotestosterone and

testosterone differed by less than 5%. On most samples tested, about 50 femtomol 5- α -dihydrotestosterone were bound per mg of cytoplasmic extract.

To demonstrate unequivocally that androgens were binding to a unique receptor site different from the oestrogen receptor also found in these cells^{6,11}, the cross competition studies outlined in Table 3 were performed. As shown, when 3 H-oestradiol is incubated with cytosol and presumably bound to oestrogen receptor, oestradiol and an antioestrogenic compound (Nafoxidine) can nearly completely displace the label from receptor sites whereas androgens and the antiandrogens (R2956) do not displace oestradiol. Conversely, when 3 H-5- α -dihydrotestosterone is incubated with cytoplasmic extracts and the ability of various steroids to displace androgen from receptor is examined, nearly the reverse results are obtained. While unlabelled oestradiol shows some ability to compete with 3 H-5- α -dihydrotestosterone for binding a Nafoxidine has little binding affinity. Potent androgens easily displace labelled 3 H-5- α -dihydrotestosterone and antiandrogens also show activity. Progesterone displaces 3 H-5- α -dihydrotestosterone from its receptor but does not displace 3 H-oestradiol. 5- β -Dihydrotestosterone does not displace 3 H-5- α -dihydrotestosterone from receptor and, as shown in Table 1, does not promote thymidine or leucine incorporation. Thus, these data strongly suggest separate receptors for oestrogen and androgen in these cells.

The demonstration of androgen responsiveness of this cell line *in vitro* provides a rational basis for the response of at least some patients with metastatic breast cancer to adrenalectomy^{12,13} since this ablative procedure may remove weak androgen precursors such as dehydroepiandrosterone and androstenedione which can be converted by some human breast cancer to more potent androgens¹⁴. Furthermore, since some human breast cancers may be responsive to androgen, it is possible that an examination of human tumours for androgen receptors may help with therapy of some patients, much as oestrogen receptor studies have appreciably improved therapeutic decision making in breast cancer¹⁵. We¹⁶ and others^{17,18} have described androgen receptors in some fresh human tumour samples but their value in guiding therapy remains to be seen.

Finally, the existence of a human cell line showing severalfold stimulation by androgens in a defined system, free from the effects of other trophic hormones should provide a useful reagent for the further study of the mechanism of androgen action.

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Table 3 Ability of various steroids to displace either 3 H-5- α -dihydrotestosterone or 3 H-17- β -oestradiol from receptor in MCF-7 human breast cancer

Competitor	Molar excess	3 H-oestradiol % bound	3 H-5- α -dihydrotestosterone % bound
Control (no competitor)		100	100
17- β -Oestradiol	2	13	91
	20	8	62
	200	2	22
Nafoxidine	20	56	86
	200	19	80
	2,000	14	57
5- α -Dihydrotestosterone	2	100	11
	20	97	2
	200	98	1
R2956	20	97	17
	200	96	9
	2,000	97	4
Progesterone	20	87	73
	200	91	41
	2,000	93	34
β -Dihydrotestosterone	20	N.D.	85
	200		88
	2,000		92

Cytosols from MCF-7 human breast cancer were incubated with either 5×10^{-9} M 3 H-17- β -oestradiol or 3 H-5- α -dihydrotestosterone with or without the presence of excess unlabelled competitor as noted. After 18-h incubation at 0 °C specifically bound steroid was assayed²¹. Results are expressed as percentage of specifically bound tritiated steroid as compared with no competitor.

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Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells

NERVE growth factor (NGF) is a chemically characterised polypeptide which affects the development of the sensory and sympathetic nervous systems and is required for maintenance of sympathetic neurones¹⁻³. Its mechanism of action and its precise role in embryogenesis are unknown.

NGF promotes neurite outgrowth from embryonic sensory or sympathetic ganglia both *in vitro* and *in vivo*¹⁻³. This phenomenon has provided the basis of much NGF research, and is utilised in the most common NGF bioassay⁴. Some cells in cultured human neuroblastomas, but not in mouse neuroblastomas, also respond to NGF^{5,6}. Homogeneous populations of replicating NGF-responsive cells, however, have not been available for study. We describe here NGF-induced process formation by a transplantable rat pheochromocytoma in cell culture.

Tumour cells were obtained from a previously reported noradrendine-producing adrenal pheochromocytoma^{7,8}, which was passaged subcutaneously in New England Deaconess Hospital white rats. The tumours were excised aseptically, rinsed in several changes of Ca²⁺ and Mg²⁺-free phosphate-buffered saline, and mechanically dissociated by trituration with a Pasteur pipette. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (Gibco) which was supplemented with 10% foetal bovine serum (Flow Laboratories), penicillin (50 U ml⁻¹) and streptomycin (50 µg ml⁻¹) and were plated on polystyrene tissue culture dishes (Falcon) which in some

cases were coated with air-dried rat tail collagen⁹. Cultures were maintained at 37 °C in a water-saturated atmosphere of 92% air and 8% CO₂. The identity of the cultured cells as pheochromocytes was confirmed by electron microscopy and formaldehyde-induced fluorescence⁸ (Fig. 1e and f).

Plating efficiencies (from inocula of 250,000 cells per 35-mm dish) were determined by counts of trypsinised cells or by measurement of total protein or absorbance (260 nm) of cells dissolved in 0.1 N NaOH. Process outgrowth was quantified by direct counts of cells or clumps of cells with processes, and was studied as a function of NGF concentration (2.5S mouse salivary gland NGF)¹⁰ and of time. Some cells were grown in medium supplemented with hydrocortisone or corticosterone, at concentrations of 5-50 µg ml⁻¹.

NGF (50 ng ml⁻¹) produced an increase in plating efficiency from 50% to nearly 100% for freshly dissociated cells plated directly on plastic. On the collagen substrate, however, nearly identical plating efficiencies (~100%) were obtained with or without NGF. The cells had a marked tendency to grow in clumps, particularly when plated on collagen.

Short processes (20-30 µm) were evident within 24 h of plating, and thereafter elongated progressively. By 24 h *in vitro*, significantly more cell clumps with processes were demonstrable in cultures with NGF than in controls (Fig. 2a). By 1-2 weeks, twenty times as many process-forming clumps were present in the NGF-containing cultures (Fig. 2a). The actual difference between numbers of cells with processes was many times greater, but could not be quantified because individual cells often became obscured in rounded clumps (Fig. 1d). NGF-induced process formation followed a dose-response curve identical to that observed with primary cultures of sympathetic neurones¹¹, with concentrations of NGF as low as 0.5 ng ml⁻¹ (2×10^{-11} M) eliciting a maximum percentage of responding clumps after 24 h of treatment (Fig. 2b). Neither hydrocortisone nor corticosterone affected process formation.

Processes formed in the presence of NGF tended, after a

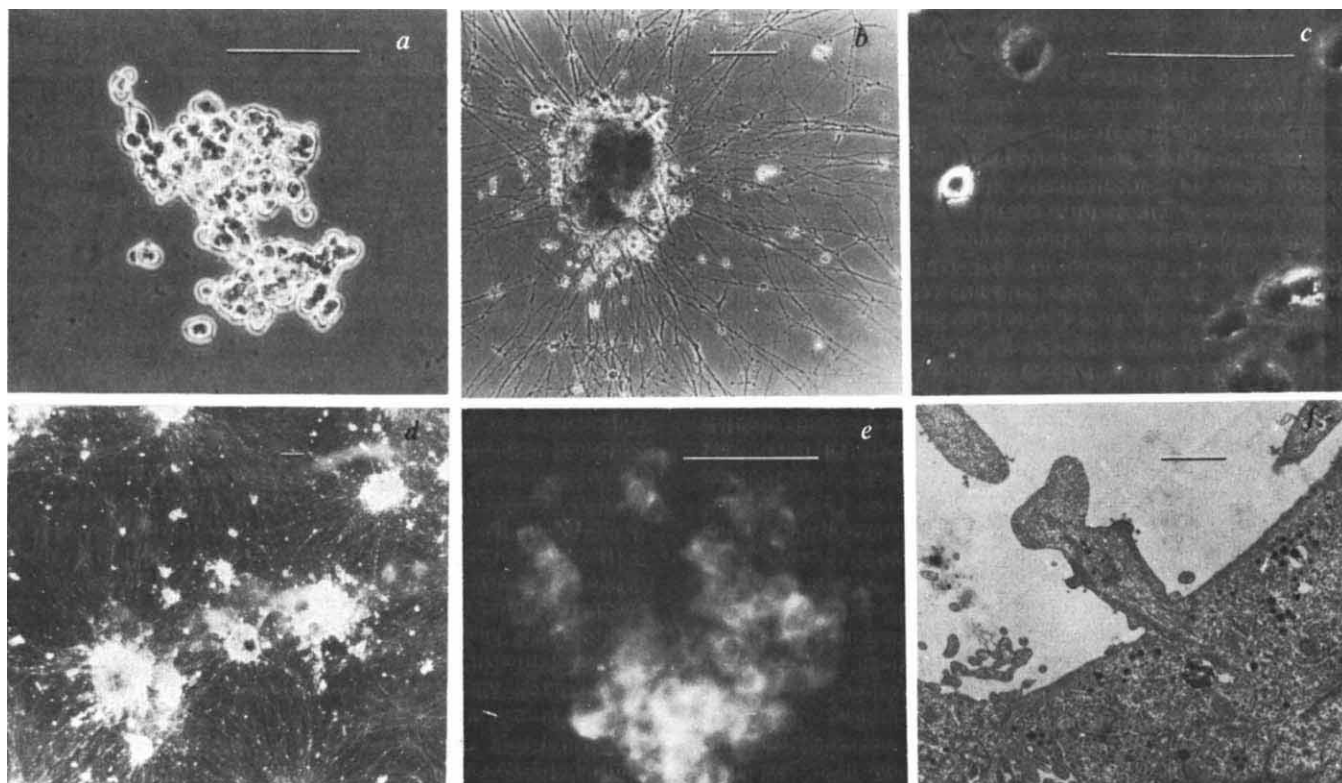


Fig. 1 Micrographs of cultured pheochromocytoma cells. *a* and *e*, No NGF; *b-d* and *f*, with NGF (50 ng ml⁻¹) in culture medium for 10 d. *a-c*, Unfixed, phase-contrast illumination; *d*, unfixed, dark-field illumination; *e*, formaldehyde-induced histofluorescence of catecholamines; *f*, electron micrograph showing chromaffin granules and part of a process. All cells were grown on a collagen-coated surface. The bar represents, in *a-e*, 100 µm, and in *f*, 1 µm.

week *in vitro*, to become longer (to $> 500 \mu\text{m}$) and more branching than in untreated cultures ($< 50 \mu\text{m}$), and many had varicosities (Fig. 1a-d). Such pheochromocytoma processes resemble those produced by sympathetic neurones in primary cultures, particularly with respect to their fineness of diameter and tendency to form fascicles^{12,13} (Fig. 1b-d). Fasciculation, in our experience, is not shown by fibres from cultured murine neuroblastoma cells. Electron microscopy (Fig. 1f) revealed processes emerging from cells with typical noradrenaline-type chromaffin granules, and the proximal segments of these processes were comparable with those in similar regions of sympathetic neurones¹⁴.

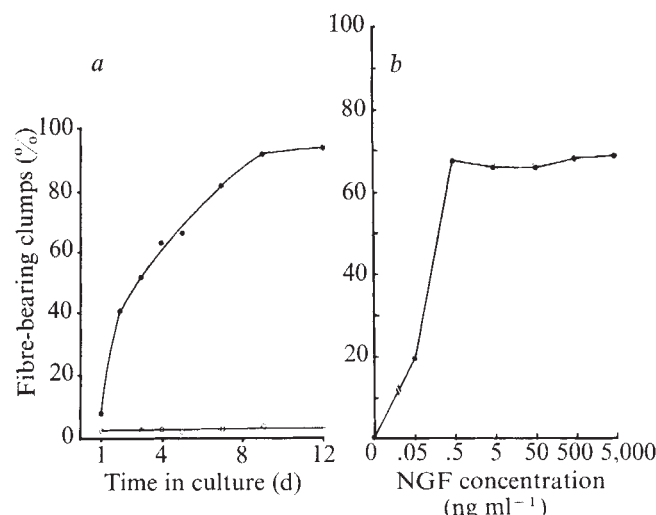


Fig. 2 Proportion of fibre-bearing cell clumps observed in cultures (a) at various times after plating in the absence (○) or presence (●) of NGF (50 ng ml⁻¹); (b), 24 h after plating in the presence of various concentrations of NGF. Cells in (a) were from a freshly dissociated tumour while those in (b) had been maintained previously for 2 weeks *in vitro* in the absence of NGF. Collagen-coated culture dishes were used. Counts were made in each case by examining the cultures with an inverted phase microscope ($\times 200$ magnification) and scoring the proportion of clumps from which processes ($> 20 \mu\text{m}$) extended. At least 300 cell clumps were scored for each count.

Tumour cells survived *in vitro* without NGF for at least 2 months. When NGF was then added to these cells, a larger percentage showed processes within 24 h than when freshly dissociated cells were treated with NGF. Although NGF-responsive cells were able to propagate, they grew extremely slowly. After approximately a month *in vitro*, cultures of pheochromocytoma cells started to become overgrown with epithelial-like and fibroblastic cells which did not produce catecholamines, or show NGF effects on plating efficiency or morphology. We are attempting to clone and propagate NGF-responsive pheochromocytoma cells.

Unlike sympathetic neurones, pheochromocytes are not generally thought to produce processes in response to NGF or to be affected by antibody to NGF^{15,16}. Neither normal nor neoplastic pheochromocytes have long processes *in vivo*, in spite of the probable ubiquity of NGF produced by fibroblasts¹⁷. Process outgrowth, however, has been described from explants of human foetal adrenal medulla¹⁸ and from cultured human pheochromocytoma cells in one of two studies^{19,20}. We have now shown that this phenomenon, in a rat pheochromocytoma, is inducible by NGF. Process formation that occurs in cultures in the absence of added NGF might reflect another mechanism, or might be attributable to fibroblast NGF or NGF synthesised by pheochromocytes themselves.

One interpretation of our findings is that pheochromocytoma cells exhibit properties of both pheochromocytes and neurones as a manifestation of neoplasia, having partially regained the pluripotency of a more primitive neural crest cell. Another possibility is that all pheochromocytes have some capability to

form long processes when removed from the presence of inhibitory substances which act *in vivo*. It has been postulated that glucocorticoids promote differentiation of foetal sympathoblasts into catecholamine-storing cells without processes^{21,22}. We were unable to inhibit NGF-induced process formation in the rat tumour cultures with high concentrations of hydrocortisone or corticosterone, suggesting that factors other than glucocorticoids account for the absence of processes on these tumour cells *in vivo*.

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Quantitative evaluation of concanavalin A receptor site distributions on the surfaces of specific populations of embryonic cells

MORPHOGENESIS and malignant invasion seem to be dependent on cellular migration and changes in cellular adhesiveness^{1–3}. Plant lectins such as concanavalin A (con A) have been extensively used to study the nature and distribution of lectin receptor sites on the surfaces of normal, embryonic and tumour cells. These sites may be involved in controlling cellular migration and adhesiveness^{2,4,5}.

Embryonic cells, like malignant cells, and other migratory cell types, are agglutinable with con A^{2,6–8}. We have recently shown⁹ that only the migratory population of sea urchin embryonic cells (micromeres) are agglutinable with con A and exhibit con A-induced capping of receptor sites¹⁰. Non-migratory embryonic cell types (macromeres and mesomeres) do not exhibit this increased con A receptor site mobility and are not significantly agglutinable with con A¹⁰. Thus, there seems to be a direct correlation between con A receptor site mobility^{10–15} and cellular migratory activity.

Using microfluorometry^{16,17}, we have quantitatively examined the topographical distribution of con A re-

ceptors on agglutinable and less agglutinable cell types obtained from the sea urchin embryo. Using fluorescein isothiocyanate-concanavalin A (FITC-con A) we have measured the relative fluorescence intensities of four distinct areas (Fig. 1a and b) on the surfaces of individual cells of the agglutinable and less agglutinable types. This technique facilitates quantification of fluorescence topo-

graphy of con A receptor sites on the surfaces of specific populations of embryonic cells.

Dissociated cells obtained at the 32/64 cell stage of developing sea urchin embryos (*Strongylocentrotus purpuratus*) were used. The procedures for preparation of the embryos and the dissociation into single cells have been described previously⁹. Dissociation of embryos at this cell-stage yields three cell types which are easily distinguishable by size: micromeres, mesomeres and macromeres. Of these, only the micromeres are migratory and significantly agglutinable by con A⁹. After dissociation into single cells the cell suspensions were layered on a 5–15% Ficoll gradient to separate out any cell debris, unfertilised eggs or undissociated embryos. The dissociated cells were removed from the gradient and washed twice in calcium and magnesium-free seawater (CMF-SW) containing 1% Ficoll. An appropriate preparation was then made to include all cell types at a total concentration of 8×10^6 cells ml^{-1} of CMF-SW. FITC-con A containing no free FITC and supplemented with 0.001 M calcium chloride was obtained from Miles-Yeda Laboratories. A sample (1 ml) of the cell suspension was treated with $750 \mu\text{g ml}^{-1}$ of FITC-con A, incubated at 17°C for 10 min, followed with 4% formaldehyde (prepared in CMF-SW containing 1% Ficoll) fixation at 4°C for 20 min. The cells were then washed with CMF-SW containing 1% Ficoll, mounted on slides and observed under incident illumination with a Leitz Orthoplan phase contrast fluorescence microscope equipped with a Leitz-MPV photometer fitted with an adjustable measuring diaphragm.

The fluorescence intensity of 10 micromeres and 10 macromeres was measured¹⁷ and the mean recorded in Table 1. Overall, the macromeres had a mean fluorescence intensity (MFI) significantly higher than the micromeres ($P < 0.025$, Student's t test). When the MFI was calculated according to surface area (assuming a smooth sphere), the micromeres displayed greater fluorescence per unit area, but this difference was not significant at the 0.05 level. Although the micromeres were the highly agglutinable cell type, they did not significantly bind more FITC-con A per unit area than the less agglutinable macromere population.

Next we measured the fluorescence intensity of four distinct areas of 10 individual cells of each type to measure quantitatively any distributional differences of surface bound FITC-con A. The results of these measurements (Table 1 and Fig. 2) indicate that the micromeres displayed

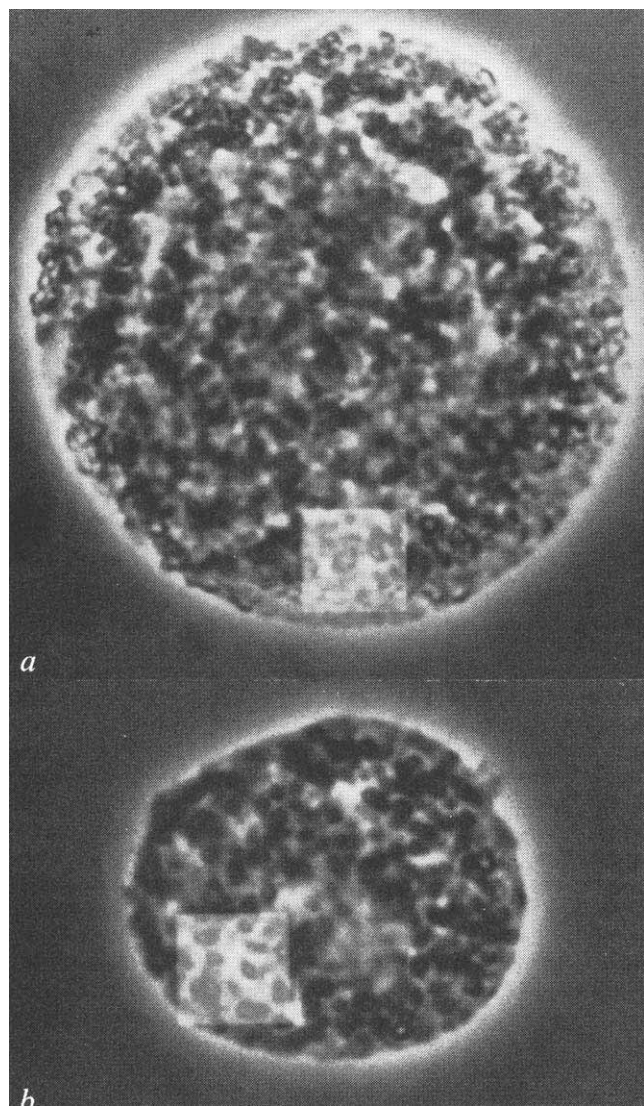


Fig. 1 Specific populations of embryonic cells showing measuring area. A sample containing cells of both populations that have been treated with FITC-con A ($750 \mu\text{g ml}^{-1}$) and fixed in 4% formaldehyde as described in the text. An individual cell, of either type, was brought into focus by phase-contrast illumination before photoexcitation to limit fluorescence fading to a minimum. At this time diameter measurements were taken, the surface area calculated and entered in Table 1. The measuring field diaphragm for fluorescence quantitation was then adjusted to cover an area of $56.25 \mu\text{m}^2$. Thereafter, the only light reaching the photomultiplier was that which was included by the measuring area only. To determine the topographical distribution of FITC-con A on the surface of individual cells, the fluorescence intensity of four distinct areas comprising $56.25 \mu\text{m}^2$ were measured. The source for fluorescence excitation was from an XBO 150 W, 20 V Xenon lamp (Osram) powered by a Leitz power supply rated at $\pm 0.1\%$ constant voltage supply. The following light filters were used: excitation filter 5 mm BG12; red suppression filter 4 mm BG38; and suppression filters K495 and K510. The photomultiplier used was an EMI 9558 with photocathode type S20. The high tension supply unit was set to deliver 1.1 kv to the photomultiplier, the digital picoammeter set at a display rate of 1 s^{-1} . (ref.17). *a*, Macromere with measuring area (phase contrast $\times 1,625$); *b*, micromere with measuring area (phase contrast $\times 1,625$). All measurements of fluorescence intensity have been represented in Table 1 and Fig. 2.

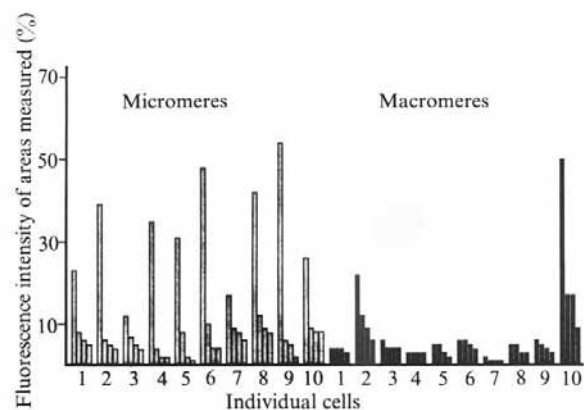


Fig. 2 Fluorescence intensity distribution of four areas measured on ten individual micromeres (shaded columns) and macromeres (solid columns). Abscissa: ten individual cells of both types. Ordinate: fluorescence intensity (%) of areas measured was derived from (see legend to Table 1):

$$\frac{\text{Intensity for each area measured}}{\text{Fluorescence intensity of individual cell}} \times 100$$

Table 1 Quantitative comparison of fluorescence topography displayed on the surfaces of specific populations of embryonic cells treated with FITC-con A

Cell type	Mean surface area (μm^2)	MFI*	MFI per surface area (μm^2)	MFI per area measured	MFI of areas measured excluding cap areas	MFI of cap areas measured only	Fluorescence intensity contribution by cap areas to MFI
Micromere	1,052.35 ± 101.91 †	55.62 ± 11.64	0.058 ± 0.014	7.23 ± 1.82	3.05 ± 0.44	19.77 ± 5.66	32.2% $\pm 4.3\%$
Macromere	3,397.97 ± 307.85	130.32 ± 33.09	0.038 ± 0.008	5.97 ± 0.91	5.00 ± 0.80	8.93 ± 2.64	10.8% $\pm 4.7\%$

*For measuring the mean fluorescence intensity of 10 cells of each type, the measuring field diaphragm was adjusted to provide an area which measured the fluorescence of only one cell. Corrections for fluorescence contributed by the background during these measurements have been made. For measurement of background fluorescence, three empty areas adjacent to the cell which had been previously quantified were measured, and the mean subtracted from the total fluorescence intensity. Units of fluorescence intensity are in terms of 10^{-8} A.

†An area measured for each individual cell of both populations was $56.25 \mu\text{m}^2$.

‡Standard error.

an uneven distribution of receptor sites as judged by the high fluorescence intensity in one area compared with the other three areas measured. The concentration of FITC-con A to one area of the cell indicates a highly clustered or capped distribution, by both quantitative measurements, and visual observations. The macromeres displayed a relatively even distribution of fluorescence except for two cells which seemed to be clustered or capped (Fig. 2) when measured quantitatively, but not when observed visually. These quantitative measurements can therefore detect distributional differences of surface bound FITC-con A that would otherwise be left undetected by visual examination.

The MFI of all areas measured of individual cells comprising both populations showed that an area on a micromere was 1.35 times brighter than that of a macromere. But when the brighter areas or capped regions were omitted from the calculations, the macromeres had a higher MFI per area measured than micromeres. This indicates that the clustered or capped regions of the micromeres contributed significantly (52%) to the MFI per area measured, whereas the contribution made by the brightest areas to the MFI per area measured for the macromeres was only 16%. Furthermore, the MFI capped regions of micromeres and the brightest areas of non-capped regions of macromeres (including the areas of the two capped macromeres, Fig. 2) indicated that capped regions of the micromeres had a significantly ($P < 0.005$) greater MFI than macromeres (Table 1). This suggests that the micromeres have areas on their surface membranes with a considerably greater con A receptor site density than macromeres. Comparison of the fluorescence intensity contribution made by the capped areas to the MFI of 10 cells of each cell type showed that the capped areas of the micromeres made a significant contribution of $32.2\% \pm 4.3\%$, whereas the contributions made by the macromeres was only $10.8\% \pm 4.7\%$ (Table 1). This difference is significant ($P < 0.025$).

These data show that the agglutinable migratory cell population, micromeres, displayed a highly clustered and capped distribution of con A receptor sites. The macromere population (less agglutinable cell type) showed a relatively uniform distribution of these receptors. We have demonstrated previously¹⁰ using these cell types that treatment of FITC-con A-labelled cells with α -methyl-D-glucoside resulted in a loss of visible fluorescence. This indicated that con A sites were indeed being labelled. Furthermore, if the cells were fixed with formaldehyde before treatment with FITC-con A all populations displayed a uniform distribution, suggesting that the con A molecules were responsible for drawing the receptor sites into clusters and caps.

The data obtained here using microfluorometry indicate that (1) the micromeres, being the more agglutinable cell type, do not bind significantly more FITC-con A per unit area than the macromeres and (2) con A-induced clustering

or capping is significantly higher in the micromeres as judged by the higher variation in fluorescence intensity of the four areas measured of each individual cell (Fig. 2) and by the fact that the fluorescence intensity contributions to the MFI of 10 cells by the capped areas in micromeres was higher than that of macromeres (Table 1).

We have shown that quantitative fluorescence topographical measurements can be used to determine con A receptor site distribution on individual embryonic cells of specific populations.

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Redistribution of surface-bound polyelectrolytes on erythrocytes

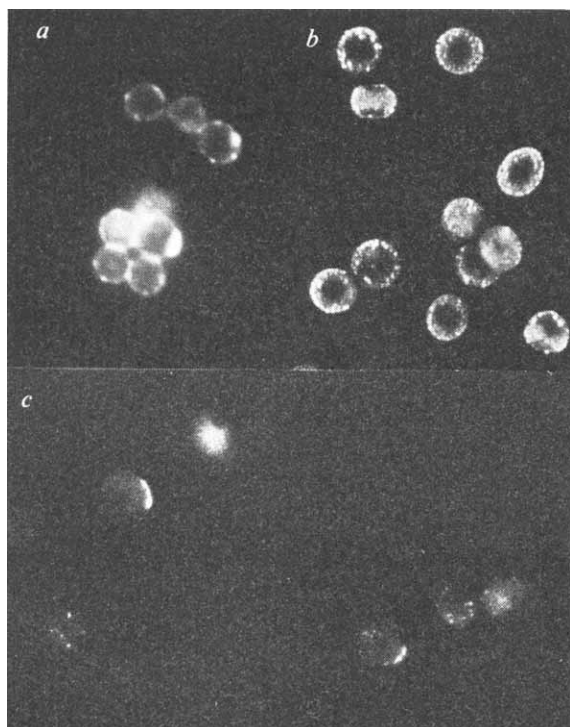
REDISTRIBUTION of the largely diffuse binding sites on the cell surface of lymphocytes and many other cell types into clusters, or into highly polarised single aggregates or caps¹, is induced by interaction with divalent or polyvalent ligands such as antibodies, lectins or ligand-conjugated macromolecules². Capping has been used as evidence for the fluid mosaic model of membrane structure³ and it has been observed in various cell types, it may be a general property of animal cells⁴. An interesting exception has been the cell surface of the adult human red blood cell,

which does not show clustering or capping with antibodies and lectins⁵. We have now demonstrated redistribution of fluorescent labelled polycations on the surface of erythrocytes fixed with glutaraldehyde or formalin when polyanions are added.

Human erythrocytes were washed four times with phosphate-buffered saline (PBS) pH 7.4, and twice with PBS containing 4% human serum albumin (PBS-4% HSA). Erythrocytes were fixed in 100 volumes of 1.5% formaldehyde in PBS for more than 3 weeks or in 2.5% glutaraldehyde in PBS containing 2mM CaCl₂ for 2 h at room temperature. Fixed erythrocytes were washed as intact erythrocytes, and suspended in PBS-4% HSA. Fluorescein-conjugated protamine and polylysine were prepared from fluorescein isothiocyanate (FITC, Sylva): 100mg protamine sulphate (uridine sulphate (PS) given by Nordisk Insulin Laboratorium, Copenhagen) or polylysine (polylysine HBr (PL) molecular weight 37,200, Miles-Yeda) in 7 ml carbonate-buffered saline, pH 9.5 was added to 0.25 mg FITC in 1 ml of acetone and incubated for 30 min at room temperature. PS-FITC and PL-FITC were purified by reprecipitation in the cold and gel filtration on Sephadex G-25.

In the presence of HSA, intact erythrocytes were labelled with PS-FITC and PL-FITC within a few minutes and uniformly diffuse labelling was observed on 20–30% of them. Erythrocytes fixed with formalin or glutaraldehyde were 100% labelled in the same homogeneous way as the labelled intact cells. The covering of polycations was removed by one or two washings with PBS-4% HSA after which no fluorescent labelling was seen, but in the incubation medium the fluorescent surface coating was stable and unaltered after 24 h at room temperature. Incubation of erythrocytes in PBS without HSA gave immediate haemolysis with PL-FITC and slower haemolysis with PS-FITC. Red cell ghosts produced by polycation interactions, hypotonic conditions⁶ or addition of minute amounts of saponin exhibited patching as distinct, rather uniform spots in the presence of PS-FITC and PL-FITC (Fig. 1a).

Fig. 1 *a*, Membrane fluorescence of human erythrocyte ghosts suspended in PBS 10 min after addition of PL-FITC (100 μ g ml⁻¹) at room temperature. *b*, Intact human erythrocytes in PBS-4% HSA first incubated for 20 min with PS-FITC (100 μ g ml⁻¹) 2.5×10^7 cells per ml and subsequent addition of dextran sulphate (1.0 mg ml⁻¹). *c*, Glutaraldehyde-fixed human erythrocytes as in (*b*). Cells were examined with a Leitz Ortholux fluorescence microscope fitted with a HBO 200-W mercury arc lamp and BG12 + UG1 excitation filters.



The uniformly distributed fluorescence label of polycations observed in both intact and fixed erythrocytes was redistributed after subsequent addition of the polyanion dextran sulphate (molecular weight 5×10^6 , Pharmacia). Figure 1*b* and *c* shows the effect of dextran sulphate in a final concentration of 1 mg ml⁻¹ on intact and fixed erythrocytes respectively, previously treated with PS-FITC. Immediately after addition of dextran sulphate small, very fine fluorescent dots were seen evenly dispersed over the entire surface.

The brilliantly fluorescent spots grew larger, and after 1 h at room temperature they had merged into a few large patches and after a further 2–3 h many cells had only one or two spots, in some cases reminiscent of capping.

Redistribution of membrane components is generally used as evidence for the fluid mosaic model of membrane structure. Redistribution has also been described⁷ in the external membrane surface, where it is independent of membrane lipid fluidity. This concept is supported by our results showing that erythrocytes covered with a layer of polycations will, irrespective of whether the cell membrane is intact or immobilised, exhibit clustering and patching when interacted with a polyanion. Similarly, Davis⁸ showed that the addition of multiple antibodies to the lymphocyte surface physically alters the distribution of H-2 isoantigens. If, for example, ferritin-conjugated alloantibody is bound to the lymphocytes, it is uniformly distributed over the cell surface. Subsequent interaction with ferritin antibodies causes coalescence of the ferritin conjugates into discrete zones of the cell surface. Multiple layer techniques for immunological detection of cell surface sites may therefore result in redistribution.

Interaction of polybases with erythrocyte ghosts has been studied by Katchalsky *et al.*⁹. Electron microscopy revealed that the polybases join neighbouring parts of the membrane (surface agglutination), resulting in distortions and eventually the formation of holes. This would explain the haemolytic effect of PL-FITC and PS-FITC. The clustering of fluorescent spots in our experiments may be due to such surface agglutination.

The interaction of polyanions with surface-bound polycations leads primarily to the formation of polar bonds whereby reductions in repulsive potential occur. The clustering and merging of material presumably results from random movements of surface bound components of the cells. The extensive redistribution of surface-bound material due to interaction between the cell membrane and compounds with high binding affinity would explain the increased cellular uptake of albumin¹⁰ and the increased permeability to albumin induced by protamine in Congo-red gelatine membranes¹¹.

This report calls attention to the possibility that redistribution of material bound to the cell surface can occur even when membrane proteins are immobilised. Observations of capping cannot therefore be taken as unequivocal evidence for lateral mobility of the membrane constituents.

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Erythrocyte morphology and clustering of fluorescent anti-A immunoglobulin

PLASMA membranes of many cells are fluid, with the protein and lipid components mobile in the plane of the membrane^{1,2}, as changing distribution patterns of fluorescent labelled lectins and antibodies bound to cell surfaces have shown³⁻⁵. Such observations have not been made with intact human erythrocytes, which are thought to be an exception⁶⁻⁷. Ferritin-labelled concanavalin A (con A)⁸ and soybean agglutinin (unpublished results of C. Kuettner, A. Staehlin, and J.A.G.) have been shown to be randomly arranged on the membranes of intact erythrocytes. But some clustering of con A receptors on the membranes of intact erythrocytes was shown by freeze etching when con A-coated erythrocytes were treated with anti-con A antibodies⁹. We now report evidence for clustering of fluorescein-labelled anti-blood group A antibodies on the surfaces of intact human A erythrocytes associated with alteration of erythrocyte morphology.

Defibrinated anti-A serum from Dade was precipitated with 50% saturated ammonium sulphate, redissolved in phosphate-buffered saline (PBS) and dialysed at 4 °C against PBS. The dialysed solution containing anti-A antibodies was mixed for 24 h at 4 °C with a solution of fluorescein isothiocyanate (FITC Isomer I, Sigma, about 5 mg FITC per 100 mg protein) in PBS with the pH adjusted to 9.0-9.5 by addition of trisodium phosphate. Free fluorescein was removed by dialysis against PBS at 4 °C in the dark for 1 week. The FITC conjugated anti-A (FITC-anti-A) solution was used without further dilution (anti-A haemagglutination titre of 1:512). Fresh human erythrocytes were obtained in EDTA and washed four times with PBS before use.

To obviate any difficulty in interpretation due to cell agglutination, erythrocytes were prepared for fluorescence microscopy as follows. Single drops of washed erythrocyte suspensions in PBS (about 4% v/v) were applied to glass microscope slides for 1 h at room temperature¹⁰. Non-adherent erythrocytes were removed by three washes of the slides in PBS. Adherent erythrocytes were then incubated with FITC-anti-A for 2 h at room temperature. Excess reagent was removed by three more washes with PBS.

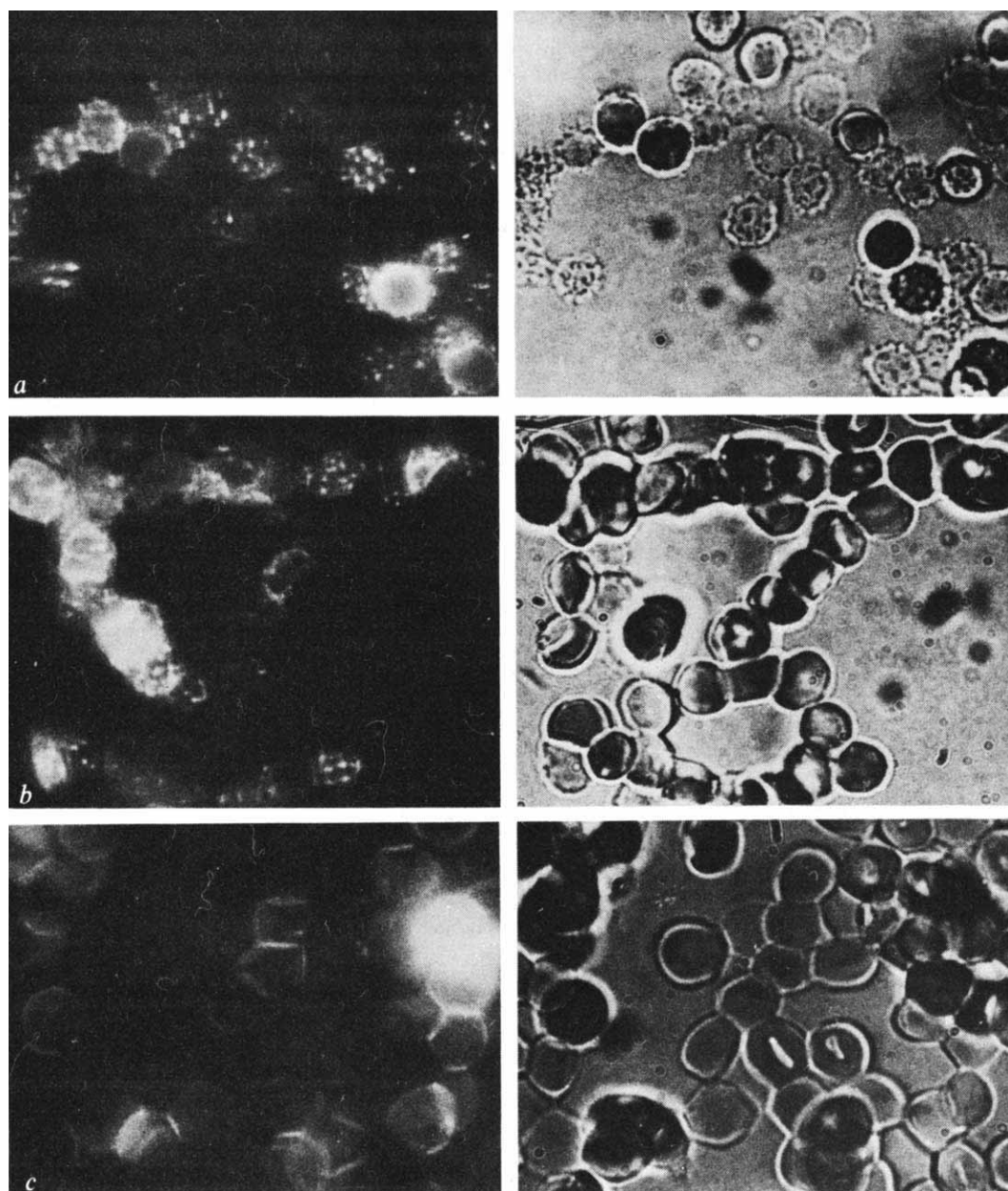


Fig. 1 Examples of fluorescent patterns obtained with human A-type erythrocytes stained with FITC-labelled anti-A globulin in several conditions. Fluorescent and phase photomicrographs of identical fields are adjacently placed. *a*, Cells which have been washed, stained and washed in PBS. Disk shape of cells converted to crenated form at staining step (line 1 in Table 1). *b*, Cells which have been washed and stained in PBS (like above to give crenates) and the morphology restored by final washes in PBS containing 1% BSA (line 2, Table 1). *c*, Cells which have been washed, stained and washed in PBS containing 1% BSA biconcave cell shape never was crenated (line 3, Table 1).

Table 1 Morphology and fluorescent patterns of FITC-anti-A-treated human erythrocytes

Morphology during preparative stages			Fluorescent pattern	
Initial washes	FITC-anti-A	Final Washes	Final observation medium	
Rough disk (PBS)	Crenate (PBS)	Crenate (PBS)	Crenate (PBS)	Stippled
Rough disk (PBS)	Crenate (PBS)	Crenate (PBS)	Disk (B)	Stippled
Disk (B)	Disk (B)	Disk (B)	Disk (B)	Uniform
Rough disk (PBS)	Crenate (PBS)	Crenate (PBS)	Rough disk (V)	Stippled
Cup (V)	Rough disk (V)	Disk (V)	Disk (V)	Uniform
Crenate (S)	Rough disk (B)	Disk (B)	Disk (B)	Uniform

A disk is a cell with the usual biconcave disk shape. A rough disk is a discoid cell with a few rounded crenations. A crenate is a spherical cell with many fine spikey crenations. A cup is a hemispherical cell with a single exaggerate concavity. These first three morphologies represent stages of a spectrum of morphological changes usually referred to as the diskocyte-echinocyte transformation⁹. B, 1% BSA in PBS; V, 0.02 mM vinblastine in PBS; S, 0.005 M sodium salicylate in PBS. Fluorescent patterns are fully described in the text.

After addition of a drop of PBS and sealing of coverslips slides were examined with Zeiss and Leitz microscopes equipped with ultraviolet epi-illumination. PBS containing 1% bovine serum albumin (BSA, Metrix) or 0.02 mM vinblastine (Lilly) was substituted for PBS in some experiments.

The FITC-anti-A globulin labelled blood group A but not B or O erythrocytes. The specificity of the fluorescence was demonstrated by showing a reduction in fluorescence intensity by preincubation of erythrocytes with unlabelled anti-A but not with anti-B blood typing serum.

The fluorescent patterns were of two general types, designated uniform and stippled. Uniform fluorescence had a uniform intensity distributed over the entire erythrocyte; a rim of fluorescence was observed where the membrane was viewed tangentially. Stippled fluorescence was of greater intensity, discretely localised to multiple (14 ± 4 per cell image) small areas about $0.5 \mu\text{m}$ or less in apparent diameter, superimposed on a dimmer uniform fluorescence of the erythrocyte surface. A tenfold dilution of the FITC-anti-A globulin reduced the total fluorescent intensity without altering the stippled pattern.

Erythrocytes washed, stained, washed and observed in PBS were extensively (finely) crenated and had stippled fluorescence, which was most intense on the tips of the spicules (Fig. 1a). The biconcave morphology of the crenated erythrocytes, which were labelled with FITC-anti-A, could be restored by addition of PBS containing 1% BSA¹¹; stippled fluorescence was not reversible (Fig. 1b). Cells prepared with 1% BSA in PBS at every stage of preparation showed little or no crenation and had uniform fluorescence (Fig. 1c). The use of BSA was not specific, for similar results were obtained with a very low concentration of vinblastine (Table 1); 0.02 mM vinblastine restored the crenated erythrocyte to an irregular disk.

We next examined crenation by an agent other than the FITC-anti-A label (Table 1). Extensive precrenation of erythrocytes produced by exposure to 5 mM salicylate¹² in PBS, followed by reversion to disks by washing with 1% BSA in PBS before staining with FITC-anti-A containing 1% BSA gave the same uniform fluorescence seen in the absence of precrenation.

Our results indicate that the fluorescence pattern produced by FITC-anti-A on the surface of A erythrocytes is related to erythrocyte morphology. Stippled fluorescence occurs only when the A erythrocyte becomes extensively crenated in the presence of the anti-A antibody¹³. Crenation can be minimised and stippling prevented if BSA or vinblastine is present throughout the preparative procedure. But the stippled pattern is not reversed by BSA or vinblastine after labelling even though the morphology reverts from a crenated sphere to a disk.

We believe that the stippling is due to the movement of A antigenic sites on the erythrocyte membrane, during crenation and in the presence of FITC-anti-A, to form discrete clusters of antigen and fluorescent antibody at the

tips of cellular spicules, so that fluorescence concentrates there. We have not been able to separate crenation from site clustering in the presence of anti-A globulin; precrenation did not lead to stippling if the erythrocyte was restored to a disk morphology before labelling with FITC-anti-A. Two studies provide some evidence for the movement of structure in intact erythrocyte membranes. Anti-con A antibodies caused clustering of previously randomly arranged con A on the erythrocyte surface made visible by freeze etching⁸. Chevelier's work indicates a moderate increase in the number of intramembranous particles per unit area at the apices of cellular spikes of crenated erythrocytes in the absence of erythrocyte-antibody interaction¹⁴; this supports the suggestion that crenation leads to the movement of membrane components. The irreversibility of the clustering after the reversal of shape to a more native form (Fig. 1c) could depend on the antibody cross linking of membrane sites. A reasonable interpretation of our results is that cytoskeletal elements of intact erythrocytes¹⁵⁻¹⁹ generally restrict the mobility of receptors over the membrane surface. Crenation of the cell either frees membrane components from the restriction of movement or the crenation mobilises the cytoskeletal elements so that they direct the movement of the anti-A receptors to the apices of the crenates. Our experiments provide no evidence to aid the choice between these possibilities. The mechanism by which vinblastine prevents stippling is unclear. It might be a direct effect on cytoskeletal components²⁰, an alteration of membrane proteins²¹ or a change in membrane fluidity.

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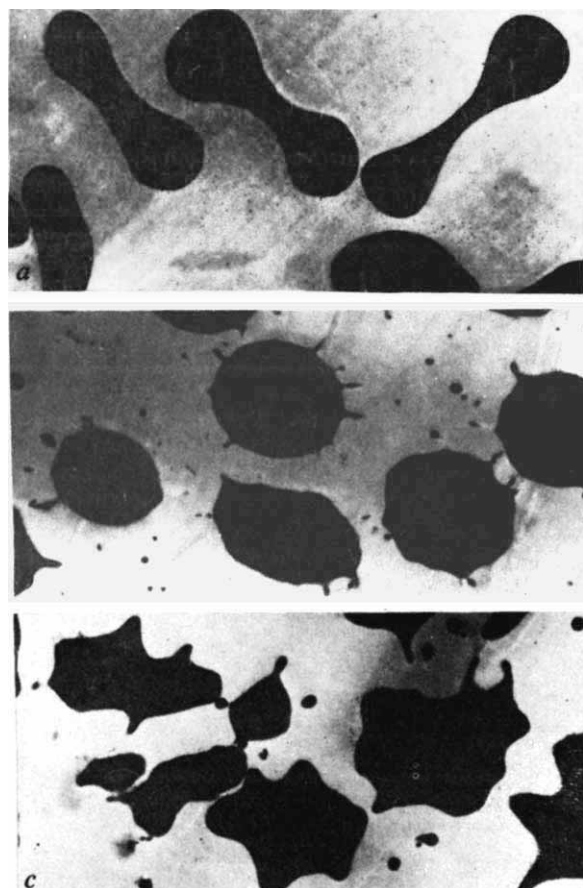
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Accumulation of 1,2-diacylglycerol in the plasma membrane may lead to echinocyte transformation of erythrocytes

THE change in shape of erythrocytes from the normal biconcave disk to a spiculated sphere (echinocyte¹) can be caused by an increased intracellular concentration of Ca^{2+} (refs 2 to 5). We have found that an immediate biochemical effect of raising intracellular $[\text{Ca}^{2+}]$ is to increase the production of 1,2-diacylglycerol and cause its accumulation in the plasma membrane. This change in membrane composition may alter the structure of the membrane and thus cause the change in shape. Energy-depleted cells also show increased 1,2-diacylglycerol and similar but less extreme morphological changes, even in the absence of Ca^{2+} .

Washed human erythrocytes ($2 \times 10^9 \text{ ml}^{-1}$) were incubated at 37°C for 1 h in HEPES-Ringer⁶ containing 11 mM glucose and bovine serum albumin 1 mg ml^{-1} (Sigma), until the cells were uniform biconcave disks. Samples (1 ml) of the cell suspension were added to test tubes containing $1 \mu\text{g}$ of the divalent cation ionophore A23187 (ref. 7) and incubated for a further 1 h. The morphological changes leading to the echinocyte form¹⁻³ were complete within 1 h when glucose was available and within only 10 min in its absence (Fig. 1). The electron micrographs (for example, Fig. 1b) suggested that membrane microvesicles were pinched off from the microvillus-like membrane processes. This was confirmed by taking the low speed supernatant after sedi-

Fig. 1 Electron micrographs showing the effect of calcium ionophore or energy depletion on human red cell morphology *a*, Normal cells; *b*, treated with A23187 for 1 h; *c*, cells incubated in medium free of Ca^{2+} and glucose for 20 h in the presence of iodoacetate, arsenate, deoxyglucose and EGTA (all 2 mM). Cells were fixed in suspension with 2% glutaraldehyde and stained with osmium tetroxide. Thin sections were prepared and examined in a Philips EM 301 electron microscope.



mentation of ionophore-treated erythrocytes and subjecting it to high speed centrifugation: electron microscopy showed that the resulting pellet consisted mainly of membrane vesicles about 100 nm in diameter.

Lipids were extracted from control and ionophore-treated cells and chromatographed^{8,9}. The most obvious change was the increased 1,2-diacylglycerol content of ionophore-treated cells (Fig. 2). Gas-liquid chromatography, carried out by Dr R. B. Watts, showed that ionophore-treated cells contained $\sim 3.5 \text{ nmol}$ of 1,2-diacylglycerol per 10^9 cells, compared with $\sim 0.5 \text{ nmol}$ per 10^9 control cells. In both cases the fatty acid composition of the diacylglycerol was similar to that of human erythrocyte phosphatidylcholine (D.A. and R.H.M., unpublished).

The total diacylglycerol production is greater than the increase noted above since in cells to which glucose was

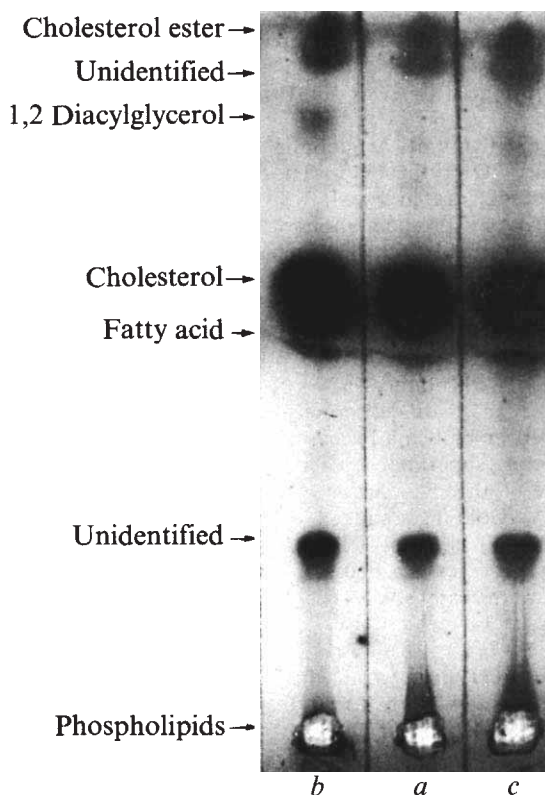


Fig. 2 Thin-layer chromatograms of neutral lipids from cells treated as in Fig. 1*a*, *b* and *c*. Lipids were separated on silica gel G plates using benzene-diethyl ether-ethanol-acetic acid (50:40:2:0.2) as described before⁸. Spots were made visible with iodine vapour and compared with standard lipids run on the same plates.

available much of the diacylglycerol generated in the early stages of ionophore treatment was converted to phosphatidate by diacylglycerol kinase, a very active enzyme in erythrocytes^{9,10}. Consequently, in erythrocytes treated with A23187 for 20 min there was a tenfold increase in the incorporation of ^{32}Pi into phosphatidate (Table 1) and a 50% increase in the phosphatidate content of the cells. The identity of the radioactive phosphatidate was established by its chromatographic behaviour and by demonstrating that when subjected to mild alkaline methanolysis it gave glycerophosphate¹⁰. Other phospholipids were separated by thin-layer chromatography¹⁰ and their phosphate contents were measured; no significant changes were observed in the concentrations of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol or sphingomyelin.

Cells treated with A23187 and a Ca^{2+} -chelator (EGTA) showed neither a change in shape nor any alteration in

diacylglycerol or phosphatidate levels, but addition of Ca^{2+} in excess of the EGTA did initiate these changes, which therefore depended on entry of Ca^{2+} into the cells.

Experiments with isolated erythrocyte ghosts showed that diacylglycerol kinase was not activated by Ca^{2+} , confirming that phosphatidate synthesis was a result of increased availability of 1,2-diacylglycerol. Ca^{2+} -stimulated production of labelled phosphatidate was observed by Redman^{11,12} in erythrocyte ghosts, but the possibility that this was secondary to diacylglycerol production was not considered.

The shape change of the ionophore-treated erythrocytes (with glucose) occurred after about 30 min, and at the same time as accumulation of 1,2-diacylglycerol. It occurred after the main period of phosphatidate labelling, suggesting that the cells could no longer phosphorylate diacylglycerol. This was confirmed when measurements of ATP levels in treated cells showed a 90% decrease in 30 min. This decrease, which was probably a result of the cell pumping out excessive intracellular Ca^{2+} , explains why glucose-deprived ionophore-treated cells were unable to phosphorylate diacylglycerol and were converted rapidly to the echinocyte form.

Table 1 Effect of A23187 on incorporation of ^{32}P into human red cell phosphatidate

	Radioactivity in phosphatidate (d.p.m.)
Control + Ca^{2+}	472
Control + EGTA	436
+ A23187 + Ca^{2+}	5,227
+ A23187 + EGTA	514

Values quoted are averages of triplicate determinations. Red cells (2×10^9) were incubated for 20 min at 37 °C either in complete HEPES-Ringer⁶ containing 2.5 mM Ca^{2+} or in Ca^{2+} -free HEPES-Ringer supplemented with 2.5 mM EGTA. Lipids were separated by paper chromatography on formaldehyde-treated papers using *n*-butanol-formic acid-water (4:1:5). In this system phosphatidate runs close to the solvent front and ahead of the other phospholipids¹⁰. The spots containing phosphatidate were cut out, digested in 72% HClO_4 and their ^{32}P radioactivity was measured by Cerenkov counting¹⁰.

The rapidity of the shape change in energy-depleted cells revived the possibility that lack of intracellular ATP may be sufficient to lead to the transformation of cells into echinocytes^{5,13}. This idea is not easily disproved, since during energy depletion intracellular Ca^{2+} and diacylglycerol concentrations will tend to rise due to lack of intracellular ATP. The following experiment, however, suggests that diacylglycerol accumulation is the most important of these three factors. Erythrocytes were incubated at 37 °C without glucose in the presence of 2 mM iodoacetate, 2 mM arsenate and 2 mM deoxyglucose, either with Ca^{2+} (2 mM) or in its absence (2 mM EGTA), and their morphology and lipid composition were examined. After 20 h they appeared spherical under light microscopy, both with and without Ca^{2+} . Under electron microscopy they resembled ionophore-treated cells except that they were less regular in shape and showed fewer surface projections (Fig. 1). There was accumulation of 1,2-diacylglycerol, even in the absence of Ca^{2+} , but this was less marked than in ionophore-treated cells (Fig. 2), as expected from the smaller morphological effects. It presumably resulted from a process different from that triggered by Ca^{2+} influx into healthy cells, possibly phosphatidate phosphohydrolase activity; this activity exists in these cells¹⁴ and is not influenced by Ca^{2+} (D.A. and R.H.M., unpublished).

Thus biconcave erythrocytes are converted to echinocytes if diacylglycerol accumulates in the cell membrane. The diacylglycerol can be produced either in the presence of increased intracellular Ca^{2+} (from phosphatidylcholine?) or during energy deprivation (from phosphatidate?). In both cases, accumulation of diacylglycerol, leading to

membrane evagination and vesiculation, will occur mainly in the inner leaflet of the membrane, since the changes occur only in response to alterations in the internal Ca^{2+} or ATP levels. This might be an effect produced by diacylglycerol accumulation in the inner leaflet of the membrane, for the addition of diacylglycerol or ceramide to the outer leaflet leads to invagination and inward vesiculation¹⁰. In both situations membrane fusion occurs in the diacylglycerol-enriched membrane face, a result consistent with the observation that externally added diacylglycerol causes fusion of avian erythrocytes¹⁵. Production of diacylglycerol may also explain the observation that intracellular Ca^{2+} facilitates cell fusion¹⁶. Ca^{2+} -stimulated production of diacylglycerol is not confined to erythrocytes, for pig lymphocytes, when incubated with A23187, Ca^{2+} and ^{32}P , showed rapid labelling of phosphatidate (data not shown). Furthermore, during muscarinic cholinergic stimulation of pancreas, when intracellular $[\text{Ca}^{2+}]$ increases, there is a pronounced rise in diacylglycerol concentration¹⁷. It therefore seems possible that Ca^{2+} -stimulated diacylglycerol production might have a role in various physiological situations where intracellular Ca^{2+} has been implicated in cell functions involving changes in plasma membrane topography, fluidity or fusibility¹⁸⁻²⁰.

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Autoradiographic localisation of α -bungarotoxin-binding sites in the central nervous system

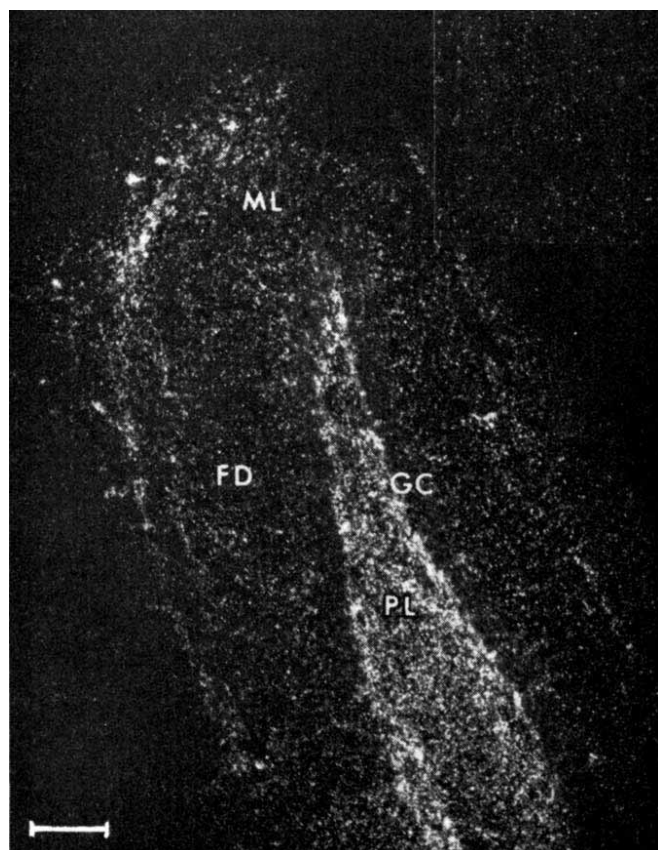
SNAKE α -toxins have high specificity and affinity for nicotinic receptors, and have been used widely for the characterisation of peripheral acetylcholine receptors of electric organs and skeletal muscle. In some cases autoradiography has shown the extent and local distribution of bound neurotoxins¹⁻⁵. Although α -bungarotoxin (α -BTX) does not pass the blood-brain barrier and therefore does not enter the central nervous system (CNS) to a measurable extent⁶, central nicotinic acetylcholine receptors may bind α -toxins *in vitro*, and several investigators have used this toxin to characterise brain receptors⁷⁻⁹. Its specificity for central nicotinic receptors has been inferred not only from its peripheral action, but also from cytological and pharmacological observations: binding activity is highest in synaptosomal preparations, and is inhibited by various

nicotinic drugs. Studies on the localisation of toxin-binding sites in brains, not so far reported, would be valuable, not only to corroborate neurotoxin specificity, but also to provide information on the regional distribution of nicotinic receptor sites in the CNS.

Using ^{125}I - α -BTX, we have found concentrations of binding sites in homogenates of whole brain comparable with receptor concentrations reported for the innervated rat diaphragm^{10,11}: approximately 1.6 pmol per g of wet tissue in adult rats, and about 7.5 pmol per g of wet tissue in the newly hatched chick. Assays performed on individually dissected brain regions revealed marked local differences in concentrations of nicotinic receptor sites in both species; high values were repeatedly obtained for the colliculi, olfactory tubercle and hippocampus of the rat, and the retina and optic tectum of the chick, whereas other areas showed intermediate, or, as in the case of rat cerebellum, little if any binding activity (J.S., unpublished). These observations prompted a study of the distribution of α -toxin-binding sites in the central nervous system by means of light microscopic autoradiography.

Anaesthetised rats (approximately 200 g body weight) and chicks (1–7 d) were perfused with saline through the left ventricle. Brains were removed, frozen in methylbutanol at -70°C and sectioned in a cryostat at -16°C . Sections of $16\ \mu\text{m}$ were collected on subbed slides and incubated at room temperature for 30–60 min in a solution of 0.1 M sodium phosphate, pH 7.4, $3 \times 10^{-9}\ \text{M}$ ^{125}I α -BTX (approximately $10^6\ \text{Ci mol}^{-1}$ depending on the date of the experiment); this toxin concentration approximately equals the concentration of toxin-binding sites in whole brain.

Fig. 1 Dark-field photograph of an autoradiograph of a sagittal section through the layer of polymorphic cells of the rat hippocampus formation. FD, fascia dentata; GC, granule cell layer; ML, molecular layer; PL, polymorphic layer. A section, $16\ \mu\text{m}$ thick, was labelled as described in the text. Exposure time was 5 d. Scale line represents $200\ \mu\text{m}$. The inset shows the polymorphic layer of an adjacent section treated with nicotine before incubation with ^{125}I - α -BTX.



Control sections were preincubated with $10^{-7}\ \text{M}$ α -BTX or $10^{-3}\ \text{M}$ nicotine for 30–60 min after which the radioactive toxin was added. The slides were washed three times for 20 min each time in 0.1 M sodium phosphate, pH 7.4, fixed in 95% ethanol, rinsed with distilled water, and dried overnight at 40°C . Dried slides were dipped in NTB 2 Nuclear Track Emulsion (Kodak), exposed at 4°C for periods from 2 to 7 d, developed, stained with cresylviolet and coverslips placed in position. In spite of on-the-slide incubation with the radioactive toxin, low background levels were routinely obtained.

Autoradiography confirms the regional distribution of radioactive α -BTX measured by biochemical techniques and takes topographical analysis to the histological level. Figure 1 shows an example of the pattern of localisation within the hippocampal formation. Most label seems to be within the polymorphic cell layer of the fascia dentata and the thin strip in the outermost part of the stratum oriens of the hippocampus. The outer molecular layer, granule cells of the fascia dentata, Ammons pyramids and fimbria fornix are unlabelled.

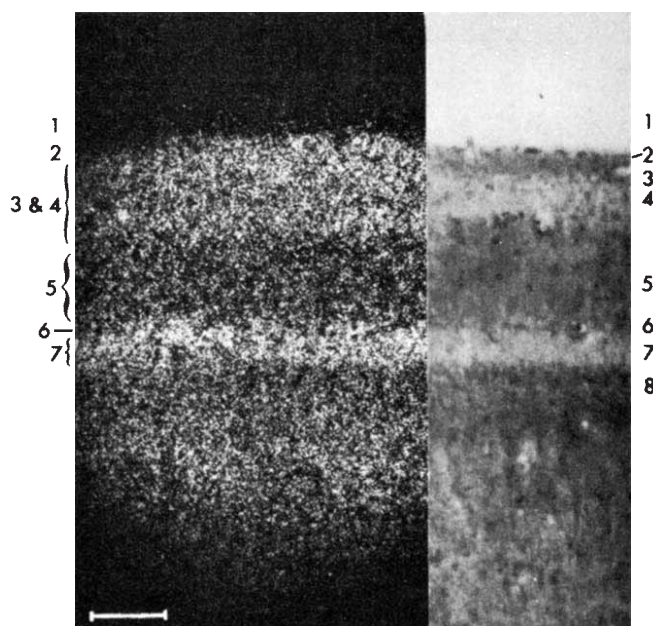


Fig. 2 Superficial layers of the optic tectum of the chick. Transverse sections show results of: Nissl stain, and acetylcholinesterase stain (modified Koelle method¹⁹) (shown on the right-hand side); and autoradiography with ^{125}I - α -BTX as described in the text (seen on the left-hand side). Exposure time was 2 d. Scale line represents $100\ \mu\text{m}$.

Homologous structures in rat and chick show similar uptake of label. Highest concentrations of toxin-binding sites occur in the optic tectum (see below), parabigeminal-isthmus nuclei, dorsal motor nucleus of the vagus, and nucleus geniculatus lateralis pars ventralis (external layer). Values are lower for thalamus, inferior colliculus and reticular formation of rat and chick, neocortex of rat and dorsal ventricular ridge of chick. A low, but significant level of binding is seen in the cerebellum whereas the caudate-putamen as well as various fibre tracts including optic, mammillo-thalamic and pyramidal tracts seem to be devoid of binding sites.

Usually, the toxin binds to areas known to contain high levels of acetylcholinesterase^{12,13}. Exceptions are observed within certain laminated structures, as shown for superficial portions of the chick optic tectum (Fig. 2). Layer 1 lacks acetylcholinesterase as well as α -toxin-binding sites. But whereas the esterase is found chiefly in layer 5, and to a lesser degree in layer 3, nicotinic receptors seem to be concentrated mostly in layers 3, 4 and 7. Esterase is virtually

absent from the latter two layers. Layer 7, which exhibits one of the highest toxin-binding levels of any brain area, contains no neurones, but radial dendrites of only the deeper-lying cells of layers 8–10. This suggests that nicotinic receptors are confined to selected segments of the dendrites.

Several regions known for their high esterase levels do not bind appreciable quantities of α -BTX. Presumably such areas contain muscarinic or other cholinergic receptors. Examples are the caudate nucleus, which according to physiological and biochemical studies contains muscarinic synapses in high concentration, and the cerebellum, which seems to lack muscarinic binding sites as well^{14–17}.

Also interesting is the high concentration of toxin-binding sites in the nucleus supraopticus. Nicotine is known to cause an antidiuretic effect by stimulating the hypothalamohypophyseal system and thereby triggering the release of antidiuretic hormone¹⁸; conceivably the nicotinic receptor sites involved in this response are among those autoradiographically visualised in the hypothalamus.

The unique localisation of α -BTX reported here is further evidence for the hypothesis that the snake α -toxins bind to central and peripheral nicotinic acetylcholine receptors with essentially equal specificity. α -BTX labelled with ¹²⁵I has been used for an electron microscopic investigation of acetylcholine receptors in the neuromuscular junction⁵. We can therefore expect that this toxin will prove useful for the study of nicotinic synapses in the CNS as well, not only at the light microscopic, but also at the ultrastructural level.

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Slowing of ionic currents in the voltage-clamped squid axon by helium pressure

PRESSURE has been shown to affect the excitability of compound and single nerves^{1–4}. The most consistent effect is a prolongation of the action potential, suggesting an influence of pressure on the kinetics of the excitation process. The physical biochemistry of pressure has been described in other systems, notably luciferin–luciferase⁵, but similar analysis for nerve action potentials proves difficult because of the complexity of the process

measured. By use of the voltage clamp⁶ more direct assessment of effects on kinetic and other excitation parameters can be made with definition of pressure effects in Hodgkin–Huxley (HH) terms⁷. We now report the effects of pressure on the behaviour of the voltage-clamped squid axon.

Experiments were carried out in a pressure chamber⁸ constructed from a commercially available high pressure pipe cross. Squid axons were dissected and mounted in an axon bath module. Incorporated into the module was a micromanipulator for electrode placement. A Peltier thermoelectric unit, used in a feedback loop with a thermistor, maintained the axon temperature to within 0.2 °C of a fixed, adjustable value (12.5 or 15 °C). Axons were bathed in flowing filtered natural seawater or potassium-free artificial seawater (430 mM NaCl, 50 mM MgCl₂, 10 mM CaCl₂, 10 mM Tris) at a pH of about 7.8. Axons were exposed to helium pressures of up to 204 atm, pressure being applied at a rate of about 7 atm min⁻¹. The voltage-clamp design followed that of Armstrong *et al.*⁹. We used “piggyback” axial electrodes internally and platinised silver electrodes with guard regions externally. A glass pipette filled with 0.5 M KCl was used for external voltage reference.

After space-clamped action potentials had been obtained, the axon was voltage clamped with holding potential set at the initial resting potential of the axon. A family of current–time curves was obtained by means of depolarising square-wave pulses. Each of these pulses was preceded by a 10-ms, 40-mV hyperpolarising pulse in order to activate sodium channels fully. Leakage currents were derived by linear extrapolation of

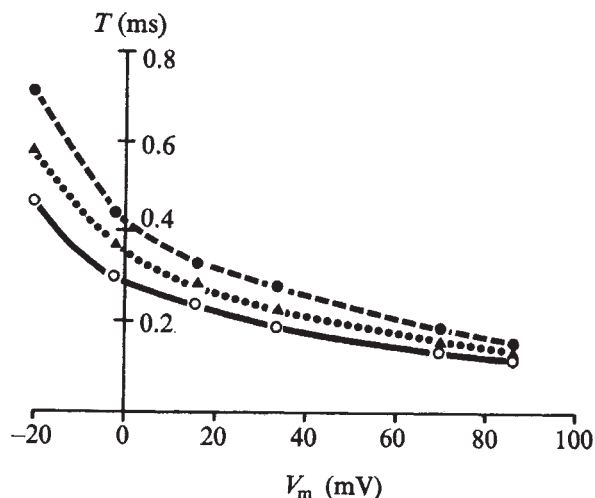


Fig. 1 Time-to-peak early current (T) plotted as a function of membrane voltage during a voltage-clamp pulse. Data were obtained at 1 atm (○), 158 atm He (●) and 79 atm He (▲), in that order. The temperature was 12.5 °C.

currents resulting from hyperpolarising pulses, and were subtracted from the observed clamp currents to obtain sodium and potassium currents. To calculate ionic conductances, these ionic currents were then divided by the difference between respective clamped membrane potentials and the observed ionic reversal potentials. The reversal potential for sodium was the clamped potential at which no sodium currents were seen; that for potassium was estimated in the unclamped axon from the maximum value of the after potential.

Effects of pressure were studied in nine axons. Action potentials obtained in the unclamped squid axon with helium pressure are comparable with those reported by Spyropoulos using hydrostatic pressure⁴; the most striking effect was a prolongation of the action potential. The amplitude of the action potential was either unaffected by helium pressure or decreased by about 10%. No significant change in resting membrane potential was observed.

In the voltage-clamped axon, pressure prolonged the time

course of both early and late currents. These effects were entirely reversible and varied with pressure, as shown in Fig. 1. Here the time-to-peak early current is plotted as a function of clamped membrane potential. Data were obtained at 1 atm, 158 atm and 79 atm, in that order. Each time to peak current was increased by pressure, and lowering the pressure from 158 to 79 atm partially restored each time toward its control value. Figure 2 shows sodium and potassium currents plotted as functions of clamped membrane potential. In this experiment a reduction in the maximum inward sodium current occurred immediately upon reaching 204 atm, with little effect on potassium currents. In two axons, non-reversible decreases in sodium reversal potential of less than 10 mV were obtained. Lastly, little or no change in the calculated maximum sodium and potassium conductances occurred. The average maximum conductances for the nine axons expressed as a percentage of control were $93 \pm 2.9\%$ for sodium and $91 \pm 4.2\%$ for potassium. The small decrease of the maximum conductances probably reflects axon deterioration in some of the preparations.

Our results with voltage-clamped squid axon show that pressure slows the time course of both early and late ionic currents. Since in most cases the maximum conductances for sodium and potassium were not significantly affected by pressure, it is likely that the number of ionic channels which open during excitation does not change and that pressure affects the kinetics of channel opening and closing; that is, the "gating" processes are primarily affected.

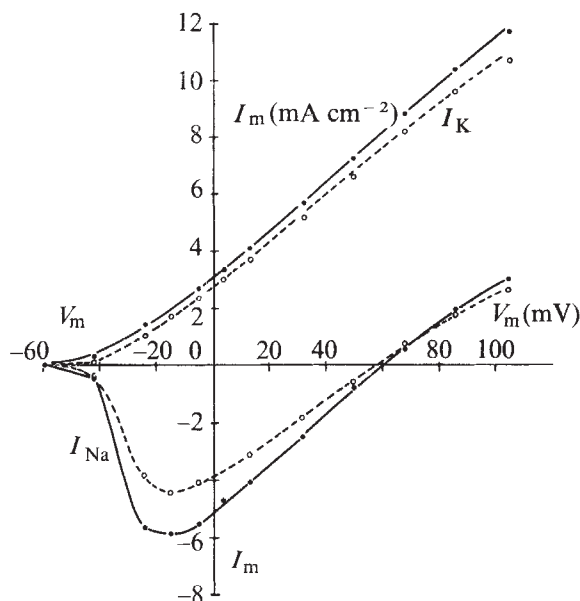


Fig. 2 Current-voltage relationship for sodium current (I_{Na}) and potassium current (I_K) at 1 atm (●) and 204 atm He (○). The temperature was 15 °C.

Further, the reduction of sodium current amplitude shown in Fig. 2 is explainable in terms of altered rate processes if we assume that gating for sodium activation is slowed but sodium inactivation is not affected by pressure. We were unable to study this behaviour after reducing pressure since sodium currents decreased further with decompression. Potassium current amplitudes also decreased by about 20% with decompression; however, we feel that these later changes are brought about by damage caused by the formation of helium gas bubbles during decompression.

To test our conclusions we carried out computer simulation, using the HH equations⁷ to generate voltage-clamp curves¹⁰ and action potentials (modified from ref. 11). By reducing the α s and β s for the sodium activation (m) and potassium activation (n) processes, but not the sodium inactivation (h) process, by about 30%, all our experimental curves were well approxi-

mated. Other combinations were tested, including alteration of the h process, but the curves obtained were not consistent with our data.

The effect of pressure on rate processes is predicted by the relation⁵

$$k_p = k_0 \exp(-P\Delta V^\ddagger/RT)$$

where k_p is a rate constant at pressure P , k_0 is the rate constant at 1 atm, $\Delta V^\ddagger$ is the volume of activation, that is, the volume change of reactants in going to the activated state, R is the gas constant and T is the absolute temperature. Assuming that the α s and β s of the HH equations represent rate coefficients for gating reactions in ionic channels, the above expression can be used to estimate the volumes of activation for the m and n processes. Although subject to large errors, these estimates, ranging from 30 to 75 cm³ mol⁻¹, lie in the range of many macromolecular reactions⁵.

A practical consideration in this study is the recently described high pressure neurological syndrome¹². Human divers and many animals subjected to helium^{13,14} or hydrostatic^{14,16} pressures develop coarse tremor, loss of sense of limb position, incoordination and disturbances of consciousness¹⁵. Although the effects on channel kinetics that we observed may be related to this syndrome, the connection is so far ambiguous since synapses are also likely to be affected by pressure¹⁶. Nevertheless, the voltage-clamp data form a basis for discussion at the molecular level of the neurophysiological effects of pressure.

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Role of PEP carboxylase during seed development in *Pisum sativum*

Pisum sativum has been put forward as a practical alternative to soybean (*Glycine max*) as a protein source that can be grown in temperate climates. In contrast to the cereals little attention has been paid to the contribution of the reproductive parts of *Pisum sativum* in the carbon economy of the developing seed. This information would be important in subsequent breeding programmes designed to exploit knowledge of the physiological components of yield.

It has been thought that the pod wall of *P. sativum* could refix, by photosynthesis, respired CO_2 from the developing seeds and then recycle it to the seeds¹. Although assimilates are obviously transferred from the pod wall to the seed, the refixation hypothesis conflicts with the estimations of pea pod photosynthesis. Pods of *P. sativum* have very low rates of photosynthesis relative to the leaves², as have those of other leguminous plants such as *G. max*^{3,4} and French bean (*Phaseolus vulgaris*)⁵. The function of the pod as a photosynthetic organ is therefore uncertain since the amount of CO_2 which could be fixed would be minimal compared with the large amount of CO_2 respired from the seed, and even this minimal fixation could only occur during the day. A more satisfactory way of reducing respiratory losses of CO_2 would be through a dark fixation system, which would work during the night and could also exist within the pea seed, which develops in perpetual low light intensity. Dark fixation systems have been described for the leaves of C3 species, for example, tobacco⁶ and barley⁷. In tobacco the initial products of this dark fixation are similar to those found in *Bryophyllum calycinum*, which has the crassulacean acid metabolism (CAM) type of photosynthetic system, depending on dark fixation of CO_2 through phosphoenol pyruvate (PEP) carboxylase. Therefore we have looked for this enzyme in extracts from developing pod walls, testae and tissue consisting of cotyledon plus embryonic axis. The activity of the photosynthetic CO_2 fixation enzyme RuDP carboxylase, was also determined using the same extracts. Isocitrate dehydrogenase (NADP-dependent) activity was assayed as this enzyme is associated with mitochondrial respiration.

PEP carboxylase activity was found in all the tissues sampled (Fig. 1). The developmental patterns of activity for this enzyme in extracts from pod walls and testae were similar, with the highest level of activity found when the tissues were youngest, the activity then decreasing as ageing proceeded. The specific activities for PEP carboxylase within the two tissues, however, were different. The maximum PEP carboxylase activity found in the pod wall was relatively low and similar to that found in the young leaves of pea, (C.L.H., unpublished) and young leaves of *Antirrhinum majus*⁸. Much greater specific activities, however, were found in the testa, the highest enzyme levels being equivalent to the PEP carboxylase activity extracted from maize plants using a

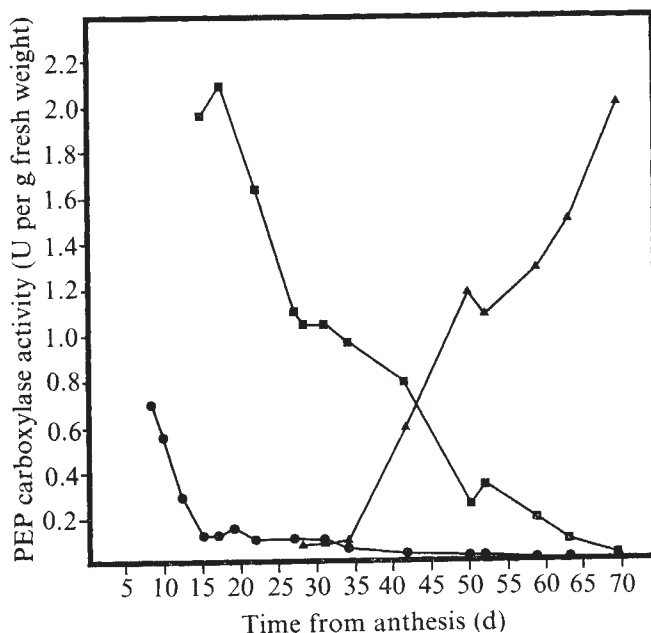


Fig. 1 Changes in the activity of PEP carboxylase extracted from pod wall (●), testa (■) and cotyledon (▲) tissue of *P. sativum*. The activity of PEP carboxylase was estimated by following the conversion of NADH to NAD in the presence of phosphoenol pyruvate at 340 nm wavelength¹³. The enzyme was coupled with malic dehydrogenase. Total reaction volume of 3.0 ml included the following: 30 μM bicine; 30 μM MgCl_2 ; 0.5 μM dithiothreitol; 4.2 U of malic dehydrogenase, and 0.25 μM NADH. The reaction was started by the addition of 3 μM phosphoenol pyruvate. The reaction mixture was buffered to pH 7.8 and the temperature was maintained at 25 °C. Enzyme activity was calculated using the extinction coefficient of NADH ($6.22 \times 10^6 \text{ cm}^2 \text{ mol}^{-1}$). The enzyme extract was prepared by homogenising 200 mg of tissue in 2 ml of buffer composed of 0.04 M Tris-(hydroxymethyl)-methylamine; 0.01 M MgCl_2 and 0.5 mM dithiothreitol, and adjusted to pH 7.5. The supernatant obtained by 20 min centrifugation at 40,000g was used as the enzyme source.

similar assay and extraction procedure (unpublished data of C.L.H.). The pattern of PEP carboxylase activity for cotyledons was opposite to that found during the development of pod walls and testae. The lowest level of activity was found when the cotyledons were young and the activity increased throughout development.

Table 1 Changes in RuDP carboxylase and isocitrate dehydrogenase during the development of the pod wall, testa and cotyledons

Days from anthesis	RuDP-carboxylase (mU per g fresh weight)			Isocitrate dehydrogenase (mU per g fresh weight)		
	Pod wall	Testa	Cotyledons	Pod wall	Testa	Cotyledons
8	40	—	—	1,900	—	—
10	80	—	—	1,300	—	—
15	40	20	—	1,080	3,040	—
18	30	10	—	1,070	4,280	—
22	40	0	—	1,080	3,050	—
28	40	0	0	1,300	3,050	960
34	5	10	0	1,350	3,400	1,120
42	0	10	0	880	2,530	1,230
50	0	0	0	680	1,910	1,210
59	0	0	20	410	1,500	660
69	0	0	40	100	350	700
Activity of young fully expanded leaf		3,320			1,620	

Enzyme extracts were prepared as described in Fig. 1. RuDP carboxylase activity was estimated by following the conversion of NADH to NAD in the presence of ribulose-1,5-diphosphate at 340 nm wavelength^{12,13}. The total reaction volume of 3.0 ml included: 30 μM bicine; 30 μM MgCl_2 ; 0.5 μM dithiothreitol; 150 μM NaHCO_3 ; 10 μM ATP; 0.5 μM NADH; 4 U of glyceraldehyde-3-phosphate dehydrogenase, and 12.5 U 3-phosphoglycerate kinase. The reaction mixture was buffered to pH 7.8 and the reaction temperature maintained at 25 °C. Enzyme activity was calculated using the extinction coefficient of NADH ($6.22 \times 10^6 \text{ cm}^2 \text{ mol}^{-1}$). Isocitrate dehydrogenase activity was estimated by following the conversion of NADP to NADPH in the presence of isocitric acid at 340 nm wavelength¹⁴. The total reaction volume of 3.0 ml included the following: 250 μM Tris-(hydroxymethyl)-methylamine; 5 μM glutathione; 12 μM isocitric acid; 125 μM NaCl ; 1 μM NADP and 12 μM MnSO_4 . The reaction mixture was buffered to pH 7.8 and the temperature kept at 25 °C. Enzyme activity was calculated by using the extinction coefficient of NADPH ($6.22 \times 10^6 \text{ cm}^2 \text{ mol}^{-1}$).

Table 2 Changes in net CO₂ uptake during the development of pea pods

Days from anthesis	Net CO ₂ uptake in the light (60 klx) (mg CO ₂ dm ⁻² h ⁻¹)	Net CO ₂ uptake in the dark (mg CO ₂ dm ⁻² h ⁻¹)
8	-1.25	-12.01
12	+1.75	-4.52
14	+2.44	-4.13
19	+1.07	-3.72
21	+0.63	-4.63
25	-0.63	-4.32
33	-1.21	-4.64
39	-0.87	-5.90
47	-1.38	-5.03
Net CO ₂ uptake of young fully expanded leaf	+31.90	-0.88

Net CO₂ uptake was measured using a multichannel infrared gas analyser system in which CO₂ concentration, light intensity and temperature could be varied independently¹⁵. Net CO₂ uptake is represented as positive and net CO₂ output as negative values. Each value in the table is the mean derived from the CO₂ exchange of at least six pods.

Isocitrate dehydrogenase activity was high during the early part of development of the three tissues (Table 1) and then decreased to reach a low level at the time of seed desiccation. The maximum specific activity found in the testae was approximately three times the activity found in either the pod wall, the cotyledons or young fully expanded leaves.

The activity of RuDP carboxylase in the pod wall (Table 1) was from 10 to 100 times less than the activity found in leaves and it was difficult to detect any activity for this enzyme in extracts of testae or cotyledons. The low level of RuDP carboxylase activity extracted from pod walls correlated with the low rates of net CO₂ uptake (Table 2), a net uptake of CO₂ being shown only during the first 21 d of pod development after anthesis.

The relatively high PEP carboxylase activity found in the testae and developing cotyledons suggests that recycling of respired CO₂ could occur at the site of origin within the seed. The positive correlation which exists, however, between PEP carboxylase and isocitrate dehydrogenase activity during the development of both the pod wall and the testa is not apparent for the cotyledons.

The main function of the PEP carboxylase synthesised in the cotyledons may therefore be in recycling CO₂ during germination, and not necessarily during the development of the seed. This suggestion is supported by the finding that the high PEP carboxylase activity in mature cotyledons is still present in dry seed and is maintained for the initial period of germination (C.L.H., unpublished). The level of PEP carboxylase in the pod wall declines before the development of the testae and cotyledons and it is unlikely therefore that the pod wall has an important role in fixing respired CO₂ from the developing seed.

There is increasing evidence therefore that PEP carboxylase, which is usually considered to be associated with C4 and CAM photosynthetic systems, is also involved in the development of plants with the C3 type of photosynthesis. In C3 plants PEP carboxylase activity seems to be highest during developmental phases when the rate of respiration exceeds the rate of photosynthesis. Much of the evidence supporting this hypothesis has come from developmental studies of the leaves of tobacco^{9,10}, citrus¹⁰ and *Antirrhinum majus*⁸. These and our observations may have implications regarding the evolution of the C4 photosynthetic system. It can be suggested that high levels of PEP carboxylase are induced when there is a net loss of CO₂. Plants which have evolved in temperate conditions would exhibit such a net carbon loss only a few specific times in development, or in particular specialised parts of the plant,

such as the developing seed. Plant species, however, which have evolved in environments which would normally induce high rates of mitochondrial respiration and photorespiration, would have a selective advantage in maintaining a system for refixing respired CO₂ for longer periods during development. C4 plants may have evolved initially by selection of variants in which the trigger for cessation of PEP carboxylase synthesis operated later in development. A late switching from a C4 to a C3 system has been suggested for leaves of the C4 plant *Portulaca oleracea*¹¹.

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Inactive complex formation between *E. coli* RNA polymerase and inhibitor protein purified from T7 phage infected cells

THE "host shut-off" function of T7 phage has been thought to result from an inhibition of the host *Escherichia coli* RNA polymerase, which transcribes host RNA and T7 early mRNA, by an early T7 protein presumably the product of gene 0.7, or the "host shut-off" gene¹⁻⁴. When T7 infection proceeds, early mRNA synthesis is shut off and the T7-specific RNA polymerase⁵, the product of an early gene of T7 (gene 1), transcribes late mRNA. Thus, "host shut-off" is another control factor involved in the early-late switch of T7 gene expression in addition to the switch of two RNA polymerases from the host to the phage-coded enzyme transcribing early mRNA and late mRNA, respectively, at different times of infection.

We reported previously⁶ that the "host shut-off" function of T7 is indeed an inactivation of the host RNA polymerase by an inhibitor protein bound to the enzyme. We found that when this inhibitor protein is removed from the inactive host RNA polymerase prepared from T7 infected cells, the core enzyme and the sigma factor are both functionally active⁶. The inhibitor protein did not inhibit T7-specific RNA polymerase or the *E. coli* RNA polymerase core enzyme⁶.

We report here further characterisations of the inhibitor protein (I protein). I protein was purified to an electrophoretic homogeneity, with a molecular weight of approximately 14,000, by Sephadex G-100 column chromatography. The inhibition of *E. coli* RNA polymerase holoenzyme by I protein was shown to result from a direct binding of I protein to the enzyme, thus blocking the enzyme from binding to template DNA and initiating RNA synthesis.

In our previous experiments⁶, we used a procedure similar to that described by Burgess⁷ to partially purify *E. coli* RNA polymerase up to the first glycerol gradient (without KCl) centrifugation step. The enzyme obtained from T7

infected cells was only 5% as active as the enzyme obtained from uninfected cells due to the association of the inhibitor to the enzyme. I protein was separated from the RNA polymerase by phosphocellulose column chromatography, and then purified by DEAE cellulose chromatography followed by glycerol gradient centrifugation. The inhibitor material after the glycerol gradient step was subjected to acrylamide-sodium dodecyl sulphate (SDS) gel electrophoresis and showed one major stainable protein with a molecular weight of about 66,000 (ref. 6).

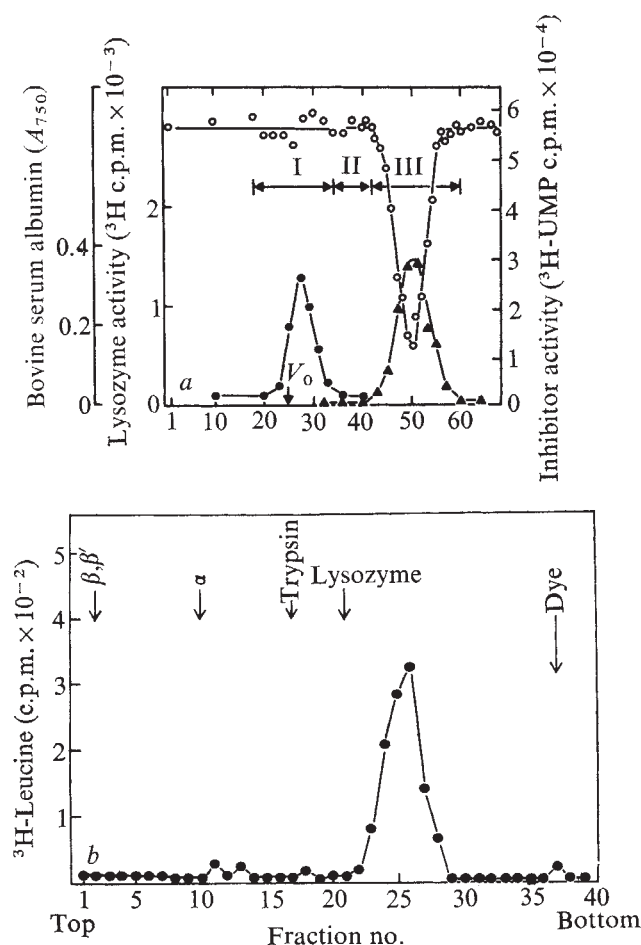


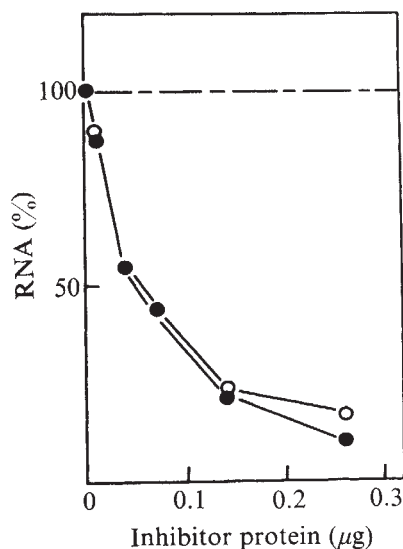
Fig. 1 *a*, Purification of I protein by Sephadex G-100 column chromatography. Sephadex G-100 column (1.2 $\times$ 45 cm) was calibrated using the following standards: bovine serum albumin (molecular weight 68,000), ovalbumin (45,000), trypsin (24,500) and lysozyme (14,500). Partially purified inhibitor protein (after the phosphocellulose and DEAE chromatography step⁶) prepared from T7 infected *E. coli* D10 F⁻ cells (labelled with ^3H -leucine (1 mCi per 8 μg per ml) for two generations before infection and with ^{35}S -methionine (950 μCi μg^{-1} ml $^{-1}$) for 9 min after infection) was applied to the Sephadex column and eluted with a 0.01 M Tris-HCl buffer, pH 7.9, containing 0.05 M KCl, 0.01 M MgCl_2 , 0.1 mM dithiothreitol, 0.1 mM EDTA and 2% glycerol. Each fraction was assayed for the inhibitory activity on *E. coli* RNA polymerase (reduction in RNA synthesis measured by ^3H -UMP incorporation in the standard assay conditions with T7 DNA⁶). Fractions designated as I, II and III were pooled, concentrated and analysed by electrophoresis (see *b*). $\circ$, Inhibitor activity, reduction in c.p.m. ^3H -UMP incorporated into RNA. $\bullet$, Bovine serum albumin, A_{750} value after the reaction of Lowry *et al.*¹⁴. $\blacktriangle$, Lysozyme, activity in c.p.m. ^3H -diaminopimelic acid assayed by the method of Schweiger and Gold¹⁵. *b*, Acrylamide-SDS gel electrophoretic profile of purified I protein. I protein was purified as described in *a* from T7 infected cells labelled with ^3H -leucine (5 mCi per 2.5 μg per ml) for 9 min after infection. Pool III material of Sephadex G-100 column fractions, similar to that shown in *a*, was subjected to acrylamide (15%) SDS slab gel electrophoresis¹⁶, and the radioactivity of ^3H -leucine in each gel slice was counted. Marker proteins, *E. coli* RNA polymerase β' , β and α subunits, trypsin and lysozyme, were electrophoresed in the same slab gel.

We found, however, that the protein which is the true inhibitor of *E. coli* RNA polymerase (the I protein) has a molecular weight of approximately 14,000 by further purifying the inhibitor material. In this experiment, a cell-free extract prepared from T7 infected cells that had been labelled with ^3H -leucine before infection and with ^{35}S -methionine for 9 min after infection was used to purify the I protein. Purification was accomplished by the use of Sephadex G-100 column chromatography instead of previously used glycerol gradient centrifugation after phosphocellulose and DEAE cellulose chromatography of the inhibitor material.

As shown in Fig. 1*a*, the activity of I protein (extent of inhibition on *E. coli* RNA polymerase) was eluted in the Sephadex elution fractions designated as pool III in Fig. 1, and the molecular weight of I protein was estimated to be about 14,000 from the molecular weights of reference proteins. No inhibitory activity on *E. coli* RNA polymerase was found in the elution fractions designated as pool I in Fig. 1 which contained a marker protein bovine serum albumin with a molecular weight of 68,000. Materials in pools I and III shown in Fig. 1*a* were concentrated and subjected to acrylamide-SDS gel electrophoresis and the radioactivity of each gel slice was measured. Pool I which had no inhibitor activity yielded a protein labelled mostly with ^3H -leucine and a molecular weight about 66,000 as the major component. Pool III showed only one protein exclusively labelled with ^{35}S -methionine.

Figure 1*b* shows the electrophoretic profile in acrylamide-SDS gel of I protein purified similarly from T7 infected cells which were labelled with ^3H -leucine for 9 min only after T7 infection. ^3H -leucine-labelled I protein was the sole component of the Sephadex elution fractions which contained the inhibitor activity (same as the result in Fig. 1*a*, pool III) and this protein migrated in the gel faster than the lysozyme marker (molecular weight 14,500). Glycerol gradient centrifugation of purified I protein, labelled with ^{35}S -methionine or ^3H -leucine, also gave an estimated

Fig. 2 Inhibition of *E. coli* RNA polymerase holoenzyme by purified I protein. Standard RNA-synthesising system⁶ contained ^3H -UTP (1 μCi per 50 nmol), 5 μg T7 DNA, or T4 DNA, or poly d(AT), and 0.85 μg purified *E. coli* RNA polymerase holoenzyme saturated with purified sigma factor¹⁷ in 0.25 ml. Purified I protein was added as indicated. ^3H -UMP incorporated into RNA after 10 min at 37 $^\circ\text{C}$ was measured. $\circ$, Poly d(AT)-directed RNA synthesis. The 100% value was 65,557 c.p.m. $\bullet$, T7 DNA-directed RNA synthesis. The 100% value was 47,351 c.p.m. T4 DNA-directed RNA synthesis was similar, and not shown. ----, Effect of I protein on RNA synthesis by T7-specific RNA polymerase on T7 DNA and by *E. coli* RNA polymerase core enzyme on poly d(AT) assayed as described previously⁶.



molecular weight of about 14,000 for I protein (data not shown). Therefore, we conclude that the I protein is a protein synthesised only after T7 infection and has a molecular weight of about 14,000.

Purified I protein inhibited the synthesis of RNA by *E. coli* RNA polymerase holoenzyme on T4 DNA, T7 DNA and poly d(AT) as shown in Fig. 2. At 50% inhibition, the molar ratio of I protein to the enzyme was about 1:1.5 (0.04 μ g I protein:0.85 μ g holoenzyme, using molecular weights 14,000 and 500,000, respectively). On the other hand, as described previously⁸, I protein showed no inhibition on the synthesis of RNA by T7-specific polymerase on T7 DNA or *E. coli* RNA polymerase core enzyme on poly d(AT).

Figure 3 shows that the inhibition of *E. coli* RNA polymerase holoenzyme by I protein is due to a direct binding of I protein to the enzyme in the absence of template DNA. Purified I protein labelled with ³H-leucine formed a tight complex with the enzyme, which was not dissociated by gradient centrifugation (Fig. 3b), with a concomitant loss in the activity of the RNA polymerase (Fig. 3a) and the inhibitory activity of I protein (Fig. 3c). I protein did not form a complex with the sigma factor when tested in a similar glycerol gradient centrifugation (data not shown).

Inhibition of *E. coli* RNA polymerase holoenzyme by I protein is limited to the initiation of RNA synthesis by the enzyme. Figure 4 shows that the effect of I protein (Fig. 4b) is almost identical to that of rifampicin (Fig. 4a) on the T7 DNA-directed RNA synthesis. Addition of I protein to the already initiated RNA-synthesising system did not stop the RNA synthesis immediately and allowed a resi-

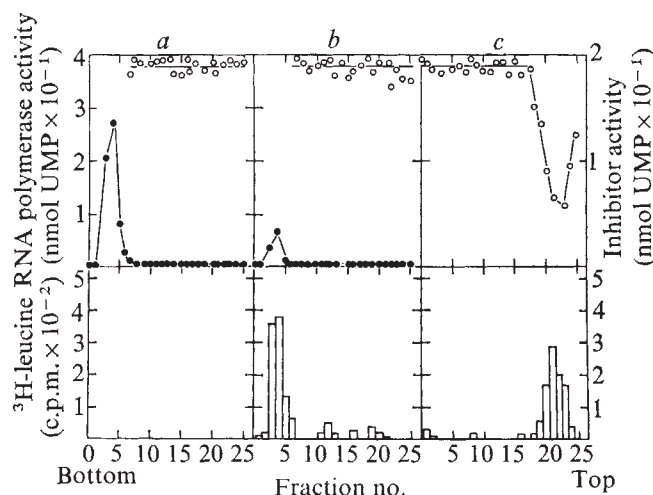


Fig. 3 Binding of ³H-leucine-labelled I protein to *E. coli* RNA polymerase analysed by glycerol gradient centrifugation. Three samples contained: a, *E. coli* RNA polymerase holoenzyme (62 μ g); b, *E. coli* RNA polymerase holoenzyme (62 μ g) and purified I protein labelled with ³H-leucine (968 c.p.m., the material shown in Fig. 1b), and c, ³H-leucine-labelled I protein (968 c.p.m.). These samples were centrifuged in 12 ml of 10–30% glycerol gradients in 0.02 M Tris-HCl, pH 7.9, 0.15 M KCl, 0.01 M MgCl₂, 0.1 mM dithiothreitol and 0.1 mM EDTA at 40,000 r.p.m. for 20 h using an SW41 rotor. Each gradient fraction (10 μ l) from a and b was assayed for the *E. coli* RNA polymerase activity in a standard system⁶ with 10 μ g T7 DNA, and the activity was expressed as nmol ³H-UMP incorporated into RNA. Each gradient fraction (30 μ l) from all three gradients was assayed for the inhibitor activity in a reaction mixture containing 10 μ g T7 DNA and 3 μ g purified *E. coli* RNA polymerase holoenzyme and plotted as a reduction in nmol ³H-UMP incorporated into RNA due to the inhibitor. The rest of each fraction from b and c was used for the measurement of acid-insoluble radioactivity of ³H-leucine after the addition of 200 μ g bovine serum albumin. ●, RNA polymerase activity, nmol UMP incorporated into RNA. ○, Inhibitor activity, reduction in nmol UMP incorporated into RNA in T7 DNA-directed RNA-synthesising system. Open columns, Radioactivity of ³H-leucine-labelled I protein.

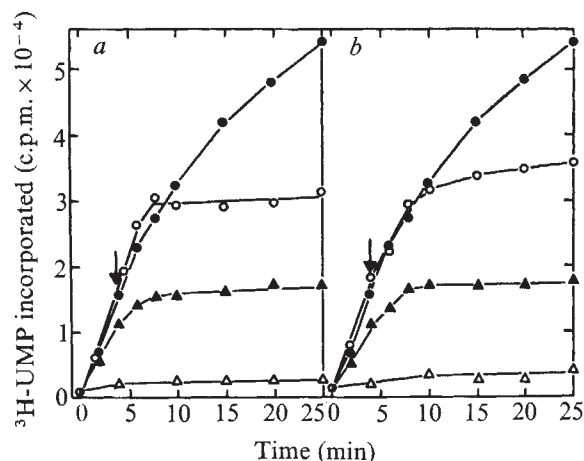


Fig. 4 Inhibition of T7 DNA-directed RNA synthesis at initiation by rifampicin (a) and I protein (b). T7 DNA-directed RNA synthesis was carried out using the standard system⁶ containing 12 μ g T7 DNA and 2 μ g purified *E. coli* RNA polymerase holoenzyme in a total volume of 2.5 ml at 37 °C. From each reaction mixture, 0.25 ml aliquots were withdrawn at indicated times and ³H-UMP incorporated into acid-insoluble material from ³H-UTP was measured. In a, 2 μ g per 0.25 ml rifampicin was added as indicated. In b, approximately 0.15 μ g per 0.25 ml of purified I protein was added when indicated. ●, Control, no rifampicin or I protein. ○, Normal reaction was started at 0 time. Rifampicin or I protein was added at 4 minutes (indicated by an arrow). Δ, RNA polymerase was incubated with rifampicin or I protein at 37 °C for 30 s, and then T7 DNA, ³H-UTP and other nucleoside triphosphates were added at 0 time. ▲, RNA polymerase and T7 DNA were incubated at 37 °C for 5 min, and then rifampicin or I protein was added together with ³H-UTP and other nucleoside triphosphates at 0 time.

dual synthesis of RNA, supposedly a completion of one round of RNA synthesis already initiated by the enzyme. If the I protein was added to the enzyme before the template DNA, the reaction was completely inhibited. If the enzyme and T7 DNA were, however, mixed before the addition of I protein and the substrates nucleoside triphosphates, one round of RNA synthesis took place.

The direct binding of I protein to the RNA polymerase described above (Fig. 3) explains the results shown in Fig. 4. A complex formed between the enzyme and I protein is inactive to initiate RNA synthesis. In addition, we found that the binding of I protein to the RNA polymerase blocks the enzyme to bind to the template DNA, as will be published elsewhere. Radioactive T7 DNA can be retained on Millipore filters if the DNA is incubated with *E. coli* RNA polymerase before filtration as described by Hinkle and Chamberlin⁸. When the RNA polymerase and I protein were incubated and then interacted with T7 DNA, DNA retention on the filter was greatly reduced.

After our previous publication⁶, Ponta *et al.*⁹ reported an isolation of *E. coli* RNA polymerase inhibitor from T7 infected cells. This protein, with an estimated molecular weight of about 14,000, inhibits the RNA synthesis by the *E. coli* RNA polymerase holoenzyme at initiation but has no effect on the T7-specific RNA polymerase and only partially inhibits the core enzyme of *E. coli*. It seems that the inhibitor protein reported by Ponta *et al.* is similar or identical to the I protein described here. Whereas Ponta *et al.* suggested that the inhibitor protein interacts with the RNA polymerase or the RNA polymerase-DNA complex⁹, we have presented evidence here that the I protein binds to the RNA polymerase in the absence of DNA and forms an inactive complex which cannot initiate RNA synthesis.

As indicated previously⁸, I protein is probably a T7 early protein. The fact that I protein is labelled exclusively by a radioactive amino acid added only after T7 infection (results in Fig. 1) supports this notion. Although T7 gene

0.7 is termed as the "host shut-off" gene³⁻⁴, cell-free extracts from the cells infected with T7 mutant phages with mutations in gene 0.7 (H3 and A57, refs 10 and 11) showed a complete inactivation of the *E. coli* RNA polymerase, that is, the "host shut-off" function was operating in these mutant phage-infected cells. At present, T7 gene 0.3 is the most likely candidate to code for the I protein, since Ratner¹² has shown that the gene 0.3 protein, with a molecular weight of 14,000, is the only T7 protein which binds to *E. coli* RNA polymerase. Although gene 0.3 has been regarded as a non-essential gene of T7 (refs 3, 10 and 11), it is interesting to note that Studier¹³ has found the gene 0.3 protein as an anti-host restriction protein. It is conceivable that a protein coded by a small phage, such as gene 0.3 protein of T7, possesses dual functions.

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Yeast mitochondrial RNA contains a short polyadenylic acid segment

POLYADENYLIC acid (poly(A)) is present at the 3' terminus of most classes of cytoplasmic messenger RNAs (mRNAs) in all eukaryotic organisms reported so far^{1,2}. Although the role of poly(A) in mRNA function and metabolism is still a subject for speculation¹, its presence has provided a convenient means for isolation of this class of mRNA by its ability to bind to cellulose and nitrocellulose filters, and to hybridise with matrices containing either oligo(dT)³ or poly(U)⁴. Most poly(A) in cytoplasmic mRNA has been found to be 100-150 nucleotides long, with an electrophoretic mobility of about 7S (ref. 1). The poly(A) segment length of yeast cytoplasmic mRNA is only 50-60 nucleotides, with an electrophoretic mobility of about 4S⁵. mRNA species with still shorter poly(A) segments of only 19-34 residues have been described in the silk moth⁶.

Mitochondrial mRNA of higher organisms also contains poly(A) at its 3' terminus. HeLa cell mitochondrial mRNA has a poly(A) segment of about 50-70 nucleotides, and presumptive mRNAs that bind to oligo(dT) have been found in mitochondria of several higher animals⁷. Therefore, the report of Groot *et al.*⁸ that they were unable to detect poly(A)-containing RNA in mitochondria of *Saccharomyces carlsbergensis* was interesting. Not only did this suggest that the relatively simple affinity chromatographic procedures using oligo(dT) or poly(U) were not

applicable to the isolation of yeast mitochondrial mRNA, but it also apparently represented another example of the similarity between mitochondria and prokaryotes. These workers suggested that addition of poly(A) to mitochondrial mRNA of higher eukaryotes was an adaptation in response to the loss of approximately 80% of the potential information in the mitochondrial genome of animals compared with yeast. Most recently, however, prokaryote RNA also has been shown to contain poly(A) segments^{9,10}.

Our laboratory has been interested in the isolation, characterisation, and translation of mRNA from yeast mitochondria. In contrast to the findings of Groot *et al.*⁸, we have been able to isolate poly(A)-containing RNA from *Saccharomyces cerevisiae* using poly(U) Sepharose chromatography. This RNA contains a short poly(A) segment of approximately 20-30 nucleotides. The small size of the poly(A) segment may account for the failure of Groot *et al.* to detect yeast mitochondrial poly(A)-containing RNA.

We used the haploid strain of *Saccharomyces cerevisiae*, D243-2B-R1 (ref. 11). Cells were grown to a mid-exponential phase and were collected as described by Casey *et al.*¹¹. Protoplasts and mitochondria were prepared according to the method of Grivell and Borst¹², except that preincubation of yeast before exposure to snail digestive enzyme (Glusulase, Endo Inc., Garden City, New York) was omitted, and protoplasts were washed only once with 1.5 M sorbitol buffer before homogenisation. The mitochondria were lysed in 100 mM NaCl, 2 mM EDTA, 10 mM Tris (pH 7.8), 0.2% sodium dodecyl sulphate containing polyvinyl sulphate (20 µg ml⁻¹) and heparin (200 µg ml⁻¹). Mitochondrial RNA was isolated from lysed mitochondria by the phenol/chloroform-isoamyl alcohol extraction procedure¹³.

A definitive fraction of the ³²P- and ³H-adenine steady-state labelled RNA isolated from yeast mitochondria is retained on the poly(U) Sepharose column under high salt conditions and is eluted with 90% formamide (Table 1). This material (fraction II) accounts for about 2.0% of the ³²P label, 6.1% of the ³H-adenine, and 5.0% of the A₂₆₀ of the total mitochondrial RNA preparation. The greater

Table 1 Fractionation of yeast mitochondrial RNA by poly(U) Sepharose 4B chromatography

RNA fraction	Recovery		
	A ₂₆₀	³² P (c.p.m. × 10 ⁶)	³ H
Total RNA	250.6	128	46
Fraction II (poly(A)-containing RNA)			
First purification	45.9	10.1	9.6
Second purification	14.1	2.9	3.1
Third purification	12.6	2.6	2.8
Poly(A) segment	—	0.019	0.058

Steady-state labelling of mitochondrial RNA was achieved by growing yeast with ³²P (orthophosphate, carrier-free, Schwarz-Mann) and 2-³H-adenine (38 Ci mmol⁻¹, New England Nuclear Corp.) to the mid-exponential phase. Mitochondrial RNA was isolated as described in the text. Poly(A)-containing RNA was isolated with poly(U) Sepharose 4B by combination of the methods of Firtel *et al.*¹⁷ and Lindberg *et al.*¹⁸. Poly(U) Sepharose was equilibrated with high salt buffer (400 mM NaCl, 2 mM EDTA, 10 mM Tris, (pH 7.5) and 0.2% sodium dodecyl sulphate). Approximately 50 A₂₆₀ U ml⁻¹ of the mitochondrial RNA preparation in high salt buffer was applied per ml of column volume at 25 °C. After 10 min, the column was washed with 9 column volumes of high salt buffer. The first four volumes of wash were pooled, and the RNA was precipitated with 2.5 volumes of ethanol (fraction I). The poly(A)-containing RNA (fraction II) was eluted with 3 volumes of formamide buffer (90% formamide, 2 mM EDTA, and 10 mM potassium phosphate, pH 7.5). The RNA was precipitated with ethanol and subjected to two further cycles of poly(U) Sepharose chromatography. Poly(A) segments were isolated from poly(A)-containing RNA after pancreatic (Worthington) and T₁ RNase (Worthington) digestion by the method of Hirsch and Penman¹³, followed by two cycles of poly(U) Sepharose chromatography.

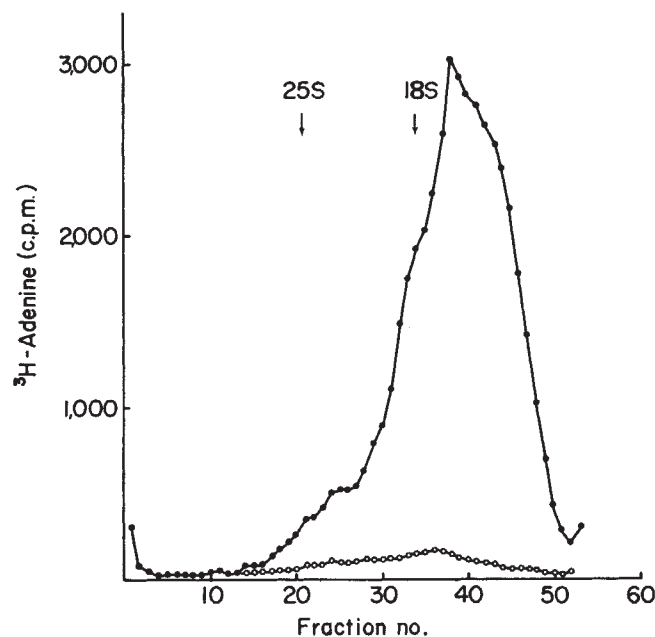


Fig. 1 5–20% sucrose gradient centrifugation of pulse-labelled yeast mitochondrial poly(A)-containing RNA. Protoplasts obtained from yeast grown to a mid-exponential phase were incubated for 15 min at 28 °C in 1.5 M sorbitol and 2% glucose containing ^3H -adenine ($20 \mu\text{Ci ml}^{-1}$). Protoplasts were washed with carrier adenine and processed as described in the text and in the legend to Table 1. The poly(A) was isolated from these protoplasts (●), and from protoplasts which had been exposed to ethidium bromide ($10 \mu\text{g ml}^{-1}$) for 10 min before incubation with ^3H -adenine (○). The RNA was layered on 5–20% linear sucrose gradient and centrifuged in an SW 25.3 rotor at 24,000 r.p.m. for 20 h. Tubes were punctured from the bottom. Fractions were counted in Instagel (Packard).

yield of ^3H compared with ^{32}P in fraction II (subsequently referred to as poly(A)-containing RNA) can be explained only in part by the poly(A) content, and is probably due to the presence of labelled polyphosphates in the total mitochondrial RNA preparation. Approximately 2% of the ^3H -adenine in the poly(A)-containing RNA was recovered after T_1 and pancreatic RNase digestion followed by two additional cycles of poly(U) Sepharose chromatography (Table 1).

The isolated mitochondrial poly(A)-containing RNA had a high molecular weight, as indicated by sucrose gradient centrifugation (Fig. 1). The sucrose density gradient profile of the pulse-labelled poly(A)-containing RNA (Fig. 1) was heterogeneous, with a modal distribution of 16S which is similar to the pattern exhibited by HeLa cell mitochondrial poly(A)-containing RNA¹³. The absence of substantial contamination of poly(A)-containing RNA by cytoplasmic RNA is supported by the observation that ethidium bromide, a specific inhibitor of RNA transcription, inhibited ^3H -adenine incorporation by more than 90% (Fig. 1). Polyacrylamide gel electrophoretic analysis under denaturing and non-denaturing conditions reported elsewhere¹⁴ also shows the poly(A)-containing RNA to be of high molecular weight, up to at least 23S, based on the electrophoretic mobility of mitochondrial ribosomal RNA. Hybridisation experiments indicate that there is little or no contamination with cytoplasmic poly(A)-containing RNA¹⁴. Specific activities of pulse-labelled poly(A)-containing RNA (fraction II) were 6–10 times greater than that of fraction I (that is RNA not retained by poly(U) Sepharose), which is consistent with a messenger role for poly(A)-containing RNA.

The electrophoretic behaviour in 12% acrylamide gels of the mitochondrial and cytoplasmic poly(A) segments isolated by RNase digestion of fraction II is shown in Fig. 2.

The mitochondrial poly(A) segment migrates much more rapidly than cytoplasmic poly(A) and almost as rapidly as dA_{10} marker. Cytoplasmic poly(A) migrates slightly more rapidly than *E. coli* tRNA marker. The electrophoretic mobility of the mitochondrial poly(A) isolated from mechanically broken cells (Fig. 2) is similar to that obtained from protoplasts, but the cytoplasmic poly(A) segment isolated from protoplasts is more heterogeneous with a somewhat more rapid modal migration (data not shown). With both isolation procedures there was little (<5%) or no contamination of mitochondrial poly(A) with material having the mobility of cytoplasmic poly(A).

An estimate of the average number of adenylic acid residues in the poly(A) segment was also derived from the ratio of AMP to adenosine of alkaline hydrolysates of the segment (Table 2); values ranging from 20 to 28 nucleotides were obtained in four different mitochondrial RNA preparations. The presence of adenosine in alkaline hydrolysates of the poly(A) segment indicates that this segment is at the 3' terminus of the poly(A)-containing RNA. The size of the segment seems to fall in the range of 20–30 nucleotides. The cytoplasmic poly(A) segment was about 50 nucleotides, which is consistent with that found by McLaughlin *et al.*⁵.

Although these results provide strong evidence that a fraction of yeast mitochondrial RNA has a poly(A) segment at its 3' end, it is conceivable that the poly(A) segment is internal and has been rendered terminal by the combined action of nucleases and phosphatase. We believe this possibility to be remote because of the high molecular weight of the poly(A)-containing RNA and the homogeneity of the poly(A) segment.

The inhibition of pulse labelling of the poly(A)-containing RNA by ethidium bromide, the distinct difference between the electrophoretic behaviour of the poly(A) segments from yeast mitochondrial RNA and that of the cytoplasmic poly(A) and the hybridisation data¹⁴ indicate that the results are not due to cytoplasmic contamination. The size of the short poly(A) segment of yeast mitochondrial RNA is similar to that observed in *E. coli*³, *Dictyostelium discoideum*¹⁵, and in silk moth chorion mRNA⁶.

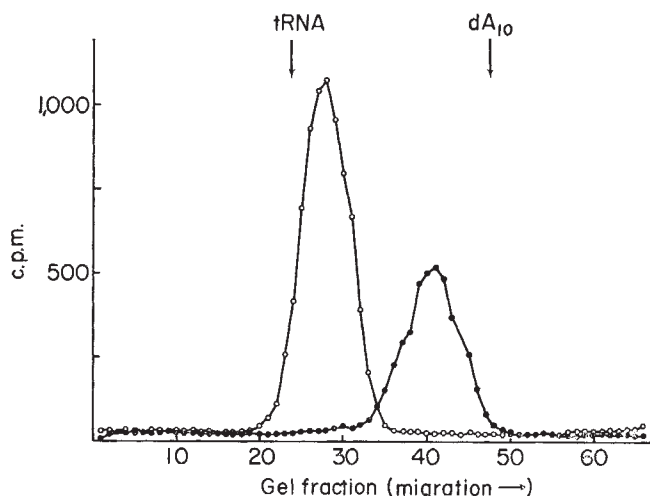


Fig. 2 Gel electrophoretic analysis of the poly(A) segment isolated from yeast cytoplasmic and mitochondrial poly(A)-containing RNA. The poly(A) segment was isolated as described in Table 1. Aliquots of 5,000 c.p.m. were electrophoresed on 12% acrylamide gels using Loening's E buffer¹⁹ at 5 mA per gel for 1.75 h at 25 °C. Gels were sliced in a Savant Autogel divider and counted in 5 ml Instagel. Internal markers of dA_{10} and yeast tRNA were used and identified by absorbance at A_{260} . The length of the poly(A) segment was estimated by the migration relative to the standards. ●, ^3H -mitochondrial poly(A) segment; ○, ^3H -cytoplasmic poly(A) segment.

Table 2 Alkaline hydrolysis of the poly(A) segment

	Mitochondrial poly(A) (c.p.m.)			Cytoplasmic poly(A) (c.p.m.)	
Preparation	1	2	3	4	4
AMP	2,200	8,725	2,063	3,641	5,870
Adenosine	96	391	93	179	115
AMP/adenosine	22.9	23.5	27.5	20.4	51.0

Four ^3H -poly(A)-containing RNA preparations, isolated as described in Table 1, were hydrolysed in 0.3 N KOH for 16 h at 37 °C. After neutralisation with HClO_4 , paper electrophoresis was carried out in 50 mM sodium formate (pH 3.5), 2 mM EDTA at 4,000 V for 2.5 h in a Gilson Electrophoretic. Nucleotide and adenosine spots were eluted and counted, or the entire electrophorogram was cut into 1-cm strips and counted in Triton X-100 liquid scintillation fluid. Preparations 1–3 were isolated from spheroplasts whereas preparation 4 was isolated from mechanically broken cells.

That the mitochondrial poly(A)-containing RNA is mRNA is suggested by pulse label experiments. We have also demonstrated¹⁴ that the poly(A)-containing RNA can direct, in an *E. coli* cell-free system, the translation of the three peptides of yeast cytochrome oxidase that have been shown to be synthesised *in vivo* by yeast mitochondrial ribosomes¹⁶.

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Mutation rate, genome size and their relation to the rec concept

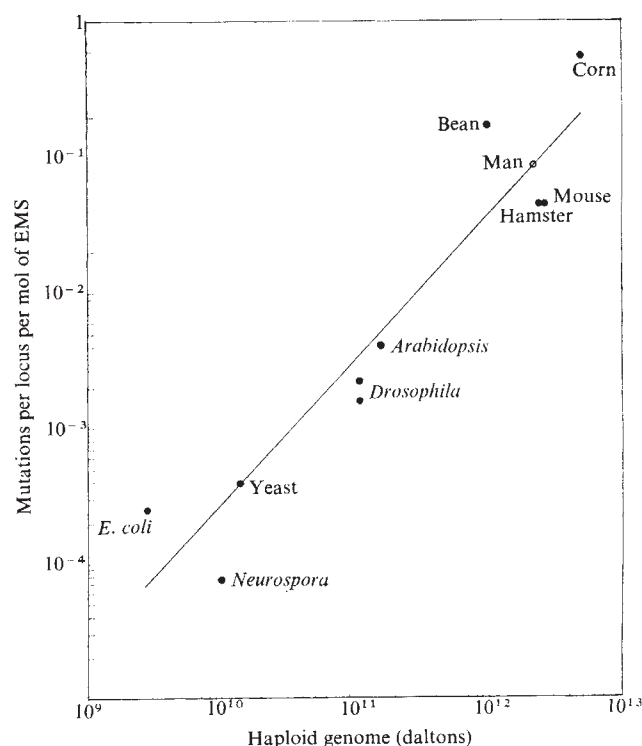
ORGANISMS vary greatly in genome size, that is, in the amount of DNA comprising the haploid genome. Rates of radiation-induced mutation expressed as mutations per locus per rad, also differ widely among organisms, but it is surprising that mutation rates and genome size are correlated¹. This correlation is unexpected because proteins are of a similar size in all organisms, implying that structural genes must also be of a similar size. Accordingly, one would expect that, although the overall mutation rate would be proportional to the overall number of genes, the mutation rate per locus would be similar in all organisms or, at least,

bear no relation to genome size. Surveying published data, however, Abrahamson *et al.*¹ found a direct correlation between rate of radiation-induced mutation and size of the haploid genome from *Escherichia coli* to *Hordeum vulgare* (the ABCW relationship). We wondered whether rates of chemically induced mutation would also be related to genome size, both because of the practical importance of being able to extrapolate to the genetic effect of a chemical on the human population and because the information might help to elucidate the mechanism underlying the ABCW relationship.

We therefore undertook a similar survey of the literature for ethyl methane sulphonate (EMS), the most widely used chemical mutagen. We were limited to studies for which we could determine the number of mutations per survivor, the number of loci at risk, the actual applied dose, and the haploid DNA content of the test organism. Dose estimation for EMS posed problems because we do not know how much EMS reached the DNA in active form: the chemical might have penetrated to the DNA in different ways in different organisms or might have been differently metabolised. Furthermore, the duration of EMS treatment varied as did the methods and conditions of the exposure. For our purposes, therefore, we assumed a uniform distribution of the chemical within the organism at the applied dose. In the case of the mouse, the injected dose of 200 mg kg⁻¹ became 200 mg l⁻¹ or 1.6 × 10⁻³ M. To provide a consistent measure of dose, it was necessary to use only concentration in all cases, rather than the product of concentration and the duration of exposure which was often unknown. The data are shown in Table 1.

To obtain the best fit of a curve representing the relationship between mutation rate and genome size, we used the method of least squares on the unweighted data or the unweighted logarithms of the data. In the former case the line of fit deviates markedly from the points for organisms with smaller genomes. Thus the logarithmic values are the only ones reported. This represents a fit to the equation $y = mx^b$ where y is the mutation rate and x is the genome size. The

Fig. 1 Rate of X-ray-induced mutation in organisms with different haploid genome sizes. The human point is not experimental.



lines shown in Figs 1 and 2 were obtained in this way. Figure 1 shows the data of Abrahamson *et al.*, for rates of X-ray-induced mutation, except that a few points have been replotted at DNA values we believe to be more accurate. The line has the parameters of $m=1.20 \times 10^{-14}$ mutations per locus per rad per dalton and $b=0.82$ ($r=0.97$). The EMS results are given in Fig. 2. In this case the line has the parameters $m=2.24 \times 10^{-14}$ mutations per locus per M per dalton and $b=1.03$ ($r=0.94$). We do not regard either of the values of b as being significantly different from 1.0 given the inhomogeneity of the data.

The relationship between mutation rate and genome size is as striking for EMS as it is for radiation, thus confirming the reality of the ABCW relation. This relation was so unexpected that we could not exclude the possibility that it was coincidence. Since the EMS data involve several organisms, loci and investigators not associated with the radiation data (although there are some of each in common), it seems most unlikely that the relationship is a statistical artefact. Accordingly it is proper to consider both the possible mechanisms that may underlie the relation and its significance for estimating human risk from chemical mutagens.

There are at least three possible ways of explaining the ABCW relationship, in which we include now EMS as well as X rays (and, presumably, many or all mutagens). These possibilities, which are not mutually exclusive, are: (1) that the target for mutation is roughly proportional to genome size, (2) that the efficiency of repair of genetic damage is inversely proportional to genome size, and (3) that the size of the mutational event is proportional to genome size. We assume, of course, that the initial lesion (for example, an alkylation) is the same but that the fate of the lesion might differ. It is conceivable, for example, that the average size of interstitial chromosomal deletions could bear some relation to genome size because two different parts of the same chromosome must be in close proximity. Thus if the condensation pattern of chromosomes resulted in proportionately longer folds or gyres in organisms with larger genomes, then the effectiveness of each lesion at producing forward mutations would increase in proportion to genome size and could account for the ABCW relationship.

The second possibility is that the effectiveness of repair is inversely proportional to genome size. This would arise if, for example, the absolute amount of repair were the same

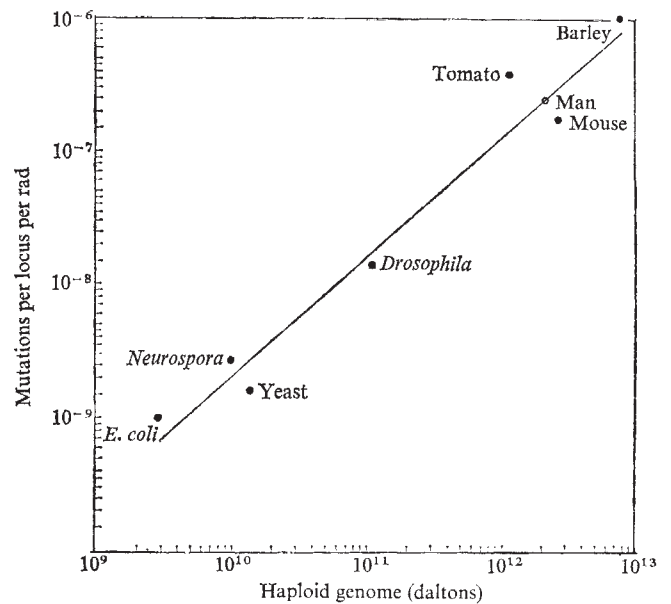


Fig. 2 Rate of ethyl methane sulphonate induced mutation rate in organisms with different haploid genome sizes. The human point is not experimental.

in all organisms (even though the total amount of damage is directly proportional to DNA content).

The remaining possibility that we have considered is that gene size differs systematically. A similar possibility has been raised, for example, from the correlation between the number and location of salivary gland chromosome bands and gene loci in *Drosophila*². Various models for the organisational and control of genes in higher organisms fit rather well with such a model in those cases where each structural gene is part of a larger genetic unit that includes controlling DNA²⁻⁴. Others have concluded that among higher organisms, specifically man and chimpanzee, the differences cannot be accounted for primarily by differences in structural genes but that differences in the controlling genes are involved⁵. Such models predict an increase in forward mutation rates with genome size since hits on controlling DNA could lead to loss of function. One test of this

Table 1 Data used to calculate the values in Fig. 2

Organism	DNA per haploid genome (daltons)	Specific locus mutation rate	Genes mutated	Dose of EMS	Duration of treatment	Specific locus mutations per mol of EMS	References
<i>E. coli</i> (bacterium)	2.8×10^9 *	$3.12 \times 10^{-5}\ddagger$ $3.8 \times 10^{-5}\ddagger$	<i>tsx</i> (resistance to phage T ₆)	0.1 M 0.2 M	8–15 min 10 min	3.1×10^{-4} 1.9×10^{-4}	*8, †9
<i>Neurospora crassa</i> (fungus)	1×10^{10} *	$7.25 \times 10^{-6}\ddagger$	<i>ad-3A</i> and <i>ad-3B</i>	9.5×10^{-2} M	60 min	7.6×10^{-5}	*10, †11
<i>Schizosaccharomyces pombe</i> (yeast)	1.4×10^{10} *	$3.9 \times 10^{-4}\ddagger$	<i>ad₆</i> and <i>ad₇</i>	1 M	2 h	3.9×10^{-4}	*12, †13
<i>Drosophila melanogaster</i> (fruit fly)	1.1×10^{11} *	$5.7 \times 10^{-5}\ddagger$	10 ³ loci for sex-linked recessive lethals†	2.5×10^{-2} M	Mutagen injected	2.3×10^{-3}	*1, †14, ‡15
<i>Arabidopsis thaliana</i>	1.6×10^{11} *	$7.8 \times 10^{-5}\ddagger$	Four loci for thiamine auxotrophy	4.1×10^{-3} M	15 h	2.0×10^{-2}	*16, †17
<i>Phaseolus vulgaris</i> (bean)	1×10^{12} *	$1.10^{-2}\ddagger$	Gene for white seed-coat colour	0.04 M	6 h	2.5×10^{-1}	*16, 18
<i>Homo sapiens</i> (man)	2.16×10^{12} *						*19
<i>Cricetus griseus</i> (Chinese hamster cell cultures)	2.51×10^{12} *	$4.46 \times 10^{-4}\ddagger$	<i>azg⁵</i>	1×10^{-2} M	2 h	4.4×10^{-2}	*19, †20
<i>Mus musculus</i> (mouse)	2.57×10^{12} *	$7.1 \times 10^{-5}\S$	Eight specific loci	200 mg kg ⁻¹ or 1.6×10^{-3} M	Mutagen injected	4.4×10^{-2}	*19
<i>Zea mays</i> (corn)	5×10^{12} *	$1.53 \times 10^{-2}\ddagger$	<i>C</i> , <i>sh</i> and <i>wx</i>	0.028 M	5 h–5 d	5.5×10^{-1}	*16, †21

Within each line asterisks, daggers and double daggers identify the appropriate references.

§ Personal communication from R. B. Cumming.

Table 2 Forward mutation rates induced in prokaryotic organisms

Organism	Genome size (daltons)	Radiation		EMS		References
		Observed values	Extrapolated values	Observed values	Extrapolated values	
<i>E. coli</i>	2.8×10^{10} **	$1 \times 10^{-9} \dagger$	6.74×10^{-10}	2.5×10^{-4}	6.75×10^{-5}	*8, †9
<i>T₂</i>	1.2×10^{10} **	$7.8 \times 10^{-13} \dagger$	3.2×10^{-11}	$7 \times 10^{-3} \dagger$	1.8×10^{-6}	*22, †23, ‡24
<i>T₄</i>	1.5×10^{10} **	$1.08 \times 10^{-8} \dagger$	4.5×10^{-11}	—	3×10^{-6}	*22, †25
ϕ X174 and S13	1.7×10^6 **	—	7.91×10^{-13}	$2.91 \times 10^{-10} \dagger$	2.38×10^{-8}	*26, †27

Asterisks, daggers and double daggers apply as in Table 1.

explanation would be to measure mutation rates in conditions such that only changes in the structural gene are detected in organisms of different genome size. Another prediction from these models is that the mutation rate will be determined mostly by the size of the structural gene when the proportion of controlling DNA is small. Thus one would expect that organisms with most of their genomes consisting of structural genes would have similar mutation rates, presumably much like that of *E. coli*. Although we have not been able to find much data on this matter, Table 2 shows that neither this prediction nor an extrapolation of the ABCW relation seems satisfactory.

The correlations, regardless of the nature of the mechanism underlying them, provide support for the proposal to express the exposure to chemical mutagens as an equivalent radiation dose. One of the major difficulties in the regulation of human exposure to mutagens is the quantitative estimation of the genetic risk. The most thorough assessment of mutagenicity has been carried out for ionising radiation and maximum population exposure limits have been laid down. On this basis a unit which could be used to quantify human exposure has been suggested, the rem-equivalent-chemical (rec or "radequiv.") which is defined as that concentration of a chemical mutagen that produces an amount of genetic damage equal to that produced by 1 rem of chronic irradiation^{6,7}. For such a unit to be meaningful the ratio of mutagenicity of the chemical to radiation must be the same for man and the test organisms. The curves in Figs 1 and 2 indicate that there is a remarkably constant relationship over several orders of magnitude of mutation rate, although there is some uncertainty in the data for any one organism. Since the lines of best fit have somewhat different slopes (1.1 and 0.9) we have used the ratio of the fitted values at the geometric mean (*Arabidopsis*) to calculate the rec value of 4×10^{-6} M. Expressed in terms of rads, therefore, treatment by a 1-M solution is equivalent to about 2.5×10^5 rad.

There are, of course, other problems in estimating the human risk and there is the problem of assessing the benefit of the chemical also. Nevertheless, if similar correlations are found for other mutagens, rough extrapolations from the simple, rapid microbiological screens to human risk can be made to provide a quantitative evaluation. This would be useful in planning mammalian experiments and evaluating the results. Mammalian systems will still be important because of the potential influence of uniquely mammalian aspects of metabolism, absorption, and excretion of compounds and the possibility of classes of damage that do not occur in prokaryotes, such as chromosome breakage and non-disjunction. Nevertheless, the rec concept has many possible uses in the light of the data presented here which suggest that there is a relatively constant value of rec for all organisms.

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Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin

It is generally agreed that B-cell surface immunoglobulin (Ig) functions as the receptor for antigen, and so accounts for the specificity of the immune response by clonal selection. It is not known, however, how the combination of surface Ig with antigen stimulates the cell to proliferation and/or differentiation to secrete antibody at a high rate¹. A signal could be delivered directly through the receptors, either by an allosteric transition in the receptor itself or by cross-linkage or aggregation of receptor Ig in the fluid cell membrane, analogous to antigen-induced or anti-IgE-induced histamine release from mast cells². Alternatively, the receptor may act passively as an antigen-specific 'address' for the delivery of an antigen-associated non-specific (that is, potentially polyclonal) signal to some other site on the cell surface³.

To test whether optimal cross linkage of receptor Ig can stimulate B cells, I have coupled purified rabbit anti-mouse Ig (predominantly anti- κ specificity) covalently to the surface of polyacrylamide beads. The anti-Ig beads were added to normal mouse spleen cell cultures. As Fig. 1 (solid bars) and Table 1 show, anti-Ig beads are strong polyclonal mitogens for normal mouse spleen cells; they stimulate a 10–20-fold increase in incorporation of ¹²⁵I-iododeoxyuridine (IdUrd) at 48 and 72 h. Control beads prepared with purified rabbit anti-*p*-azobenzenearsonate (anti-ars) antibodies had no effect (Fig. 1 and Table 1). Isotope incorporation data were confirmed by direct microscopic inspection: the number and percentage of blast cells in anti-Ig bead-stimulated cultures were ten times greater than in controls. The blasts were B cells as shown by positive immunofluorescent staining for surface Ig, while parallel phytohaemagglutinin-stimulated cultures yielded surface Ig negative blasts.

This is the first report of significant stimulation of mouse B lymphocytes by purified anti-mouse Ig. Since soluble anti-Ig

stimulates rabbit⁸, pig⁹ and chicken¹⁰ lymphocytes, the failure of soluble anti-Ig to stimulate lymphocytes of other species remains a puzzle¹. Insolubilisation on a surface has been shown to convert two other mouse B-cell ligands, which in soluble form are mitogens for T cells only—concanavalin A¹¹ and phytohaemagglutinin¹²—into B-cell mitogens, and to convert haptens or thymus dependent antigens into thymus independent antigens¹³. Insolubilisation on a surface creates a matrix of ligands at a fixed density, prevents internalisation and affects redistribution on the cell surface (although it does not prevent capping of receptors and release from the surface¹⁴). Which of these effects is crucial in this system remains to be determined.

A high density of anti-Ig on the bead surface was required for stimulation (Table 1). The peak response occurred with 64 and 130 μg of antibody per ml of beads. A concentration of 400 $\mu\text{g ml}^{-1}$ stimulated suboptimally, whereas 6 $\mu\text{g ml}^{-1}$ and 1 $\mu\text{g ml}^{-1}$ failed to stimulate. Five times fewer beads per culture gave half the response, without a change in the optimal density per bead

(data not shown). Since the beads (BioGel P30) exclude Ig from the interior, all the anti-Ig was at or near the bead surface. Beads are nearly close packed at 400 $\mu\text{g ml}^{-1}$ if one assumes the limit of a smooth spherical surface of radius 50 μm . This rather sharp dependence on ligand density is expected if the receptor Ig signal to the interior of the cell depends on the formation of small local aggregates of receptors, as seems to be the case with mast cell triggering¹⁵. To induce receptor aggregates on the cell surface, the anti-Ig molecules must be close together on the semi-rigid bead surface, since the density of adjacent pairs (or trios) of anti-Ig molecules would vary nearly as the square (or cube) of the anti-Ig density, if the distribution of anti-Ig on the bead surface is random.

Taken together with the extensive studies of the effect of density of antigenic determinants and molecular structure on the immunogenicity of thymus-independent antigens¹⁶, these results support the view that one route to B-cell activation is a critical extent and duration of cross linkage of cell surface

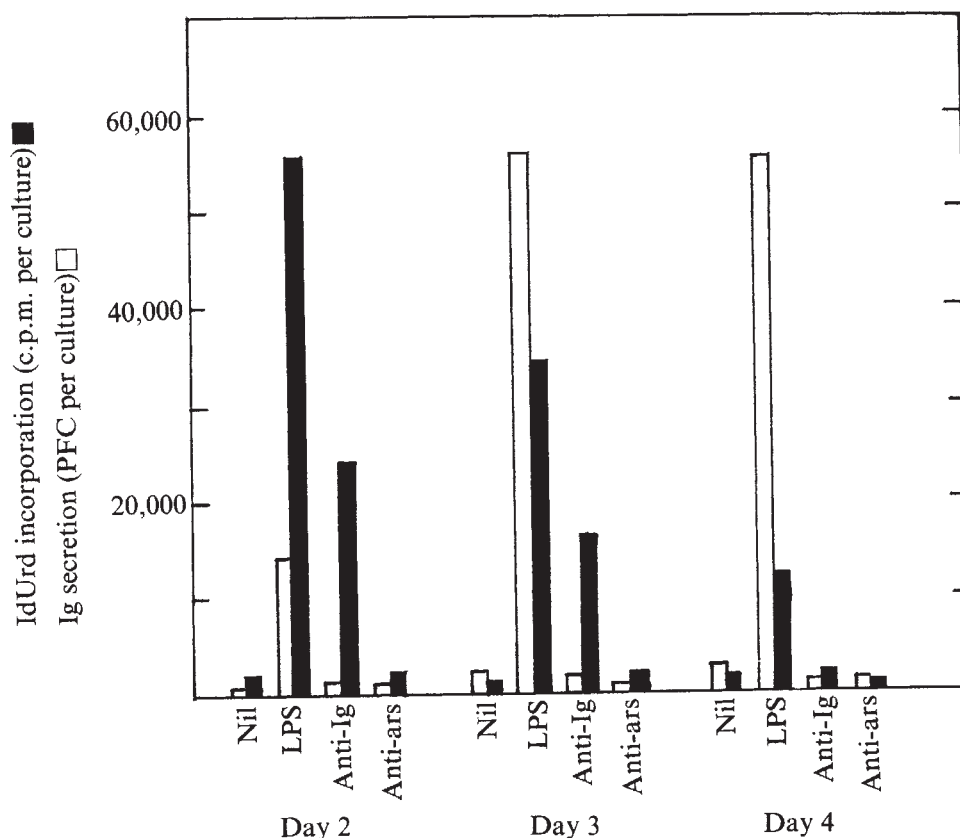


Fig. 1 Comparison of proliferation (solid bars, ^{125}I IdUrd incorporated) and number of Ig-secreting cells (open bars, PFC) in normal spleen cell cultures stimulated with lipopolysaccharide (LPS, from *E. coli* 055:B5, Difco, 100 $\mu\text{g ml}^{-1}$) or antibody-coupled polyacrylamide beads (130 $\mu\text{g ml}^{-1}$ settled beads; 5×10^3 beads per culture). Values shown are the arithmetic means of triplicate cultures. Standard errors were of the same order as those shown in Table 1. Normal 8–12-week CBA mouse spleen cells were washed with medium and cultured at 5×10^5 cells per 200 μl of modified Eagle's medium containing extra glutamine, antibiotics and 10% heat inactivated foetal calf serum in flat bottom wells in micropates (Linbro IS-FB-96) in an atmosphere of 10% CO_2 in air. 1 $\mu\text{Ci } ^{125}\text{I}$ IdUrd (100 $\mu\text{Ci } \mu\text{g}^{-1}$, Radiochemical Centre, Amersham), was added to some cultures 4 h before collection on to glass fibre filters. Parallel cultures were collected with a Pasteur pipette, washed twice, and assayed for PFC using the plaque assay described below. The anti-mouse Ig antibodies were isolated from pooled hyperimmune antisera to normal mouse Ig and to a $\gamma 2\text{a}, \kappa$ and a $\gamma 1, \kappa$ myeloma protein, using a Sepharose immunoadsorbent, and eluted with glycine-NaCl buffer, pH 2.4. The immunoadsorbent (loaned by D. Kilburn) was coupled with a mouse κ chain dimer isolated from the ascites fluid of mice bearing MOPC 70A plasmacytoma. Hyperimmune anti-ars antisera were raised in rabbits to a haemocyanin azo conjugate, and the antibodies were purified on

arsanilate Sepharose. The purified anti-Ig antibodies were trace labelled with ^{125}I by a modification of the Chloramine T technique⁴. Antibody was coupled to polyacrylamide beads (BioGel P30, 100–200 mesh, BioRad) by the acyl azide procedure of Inman and Dintzis⁵. The hydrazide intermediate (0.75 mmol g^{-1}) was reacted with 0.15 M NaNO_2 in 0.3 M HCl for 3 min. After washing as described, the beads were added to an equal volume of antibody in 0.09 M sodium tetraborate, pH 9, and tumbled slowly overnight at 4 °C. Unreacted azide groups were recon-converted to the amide. The beads were washed extensively with 6% saline pH 8.6, followed by sterile phosphate-buffered saline, pH 7.2, in which they were stored at 4 °C with azide. Ten per cent of the antibody added at 1 mg ml^{-1} beads was coupled to the beads by this procedure. Before use they were washed extensively with sterile saline and then culture medium. Immunoglobulin-secreting cells were counted using an adaptation of the reverse haemolytic plaque assay developed by Molinaro⁶. Sheep red cells were coated with anti-mouse Ig (anti- κ) using hybrid antibody⁸, and used in a monolayer plaque technique⁷. Normal spleen contained 4,000–10,000 direct PFC per 10^6 nucleated cells. In LPS-stimulated cultures, about 20% of the cells were PFC at days 3 and 4. In general, anti-Ig coated red cells gave 1,000 times the number of direct (anti-sheep red cell) plaques obtained with uncoated red cells.

Table 1 Effect of surface density of anti-Ig on stimulation

	¹²⁵ IdUrd incorporated (c.p.m. per culture $\pm$ s.e.)	Ig-secreting cells (PFC per culture $\pm$ s.e.)
Nil	1,190 $\pm$ 50	3,480 $\pm$ 260
LPS (100 μ g ml ⁻¹)	17,300 $\pm$ 540	63,300 $\pm$ 2,400
Unmodified beads	1,200 $\pm$ 80	1,690 $\pm$ 640
Anti-Ig beads 1 μ g ml ⁻¹ *	1,070 $\pm$ 30	3,480 $\pm$ 140
6 μ g ml ⁻¹	1,254 $\pm$ 30	1,720 $\pm$ 290
64 μ g ml ⁻¹	22,180 $\pm$ 1,310	5,690 $\pm$ 350
64 μ g ml ⁻¹ + LPS	17,970 $\pm$ 2,110	63,900 $\pm$ 13,140
Nil	1,350 $\pm$ 30	2,270 $\pm$ 180
LPS (100 μ g ml ⁻¹)	34,870 $\pm$ 1,350	56,130 $\pm$ 1,540
Anti-Ig beads 64 μ g ml ⁻¹	15,040 $\pm$ 1,250	1,030 $\pm$ 30
130 μ g ml ⁻¹	16,730 $\pm$ 2,390	1,450 $\pm$ 130
400 μ g ml ⁻¹	5,450 $\pm$ 770	1,120 $\pm$ 280
Anti-ars beads 400 μ g ml ⁻¹	634 $\pm$ 15	1,660 $\pm$ 280

Culture conditions were the same as Fig. 1. Cultures were collected on day 3. Arithmetic means $\pm$ the standard error of the mean for triplicate cultures are shown.

* μ g of purified antibody per ml of settled beads (10^6 beads per ml). 5×10^8 beads, enough to form a layer two beads thick on the bottom of the well, were added to each culture.

structures, one of which is receptor Ig. But two alternative explanations must be considered. The first is that the anti-Ig beads stimulate B cells indirectly, by stimulating a surface Ig-bearing, non-B cell (macrophage or activated T cell) to release a B-cell activating factor. The second is that the Fc region of the surface-bound anti-Ig delivers an activating signal to the B cell through its Fc receptors. This is unlikely since antigen-antibody complexes¹⁷ and beads densely coupled with rabbit anti-hapten antibody (Table 1) do not stimulate mouse spleen cells. These alternatives are being tested using purified B cells and beads conjugated with the (Fab')₂ fragment¹⁸ of anti-Ig. The polyacrylamide bead itself is unlikely to interact with any cell surface structure or deliver any signals to the cell. First, it is the substrate of choice for cellular immunoadsorbents since, unlike Sephadex, glass beads or other plastics, it does not adsorb sticky cells nonspecifically, as shown by 99 $\pm$ 1% yields of passed cells in column fractionation procedures¹⁹. Second, it is not a polyclonal activator as shown by normal requirements for antigen and accessory cells in the antibody response to sheep erythrocytes of mouse spleen cells cultured directly on a polyacrylamide surface in a microwell technique²⁰. Third, unmodified polyacrylamide beads or beads coated with rabbit anti-hapten antibodies failed to stimulate cells in the experiments reported here and elsewhere¹³. Sephadex G-15 beads coupled²¹ with anti-Ig at 30 μ g ml⁻¹ and 170 μ g ml⁻¹ failed to stimulate, although quantitation was difficult because the beads bound considerable ¹²⁵IdUrd in the absence of cells. The lack of stimulation may have been due to the wrong surface density of anti-Ig or to interaction of the Sephadex with the cell surface.

Although the anti-Ig beads were comparable with LPS in their ability to stimulate cell proliferation, it was striking that unlike LPS or other B-cell mitogens, with the exception of dextran sulphate²², they failed to stimulate significant increases in numbers of immunoglobulin-secreting cells (Fig. 1 and Table 1). Since the reverse haemolytic plaque assay (see Fig. 1 legend) might be relatively inefficient for IgG and IgA-secreting cells, the blasts in bead-stimulated cultures were examined for intracellular Ig using a polyvalent fluorescent anti-Ig antiserum, and found to be negative. This result has several possible interpretations: (1) The anti-Ig beads may be turning off Ig synthesis in the stimulated cells. I have found that free anti-Ig inhibits plaque-forming cells without much effect on IdUrd incorporation in LPS-stimulated cultures (data not shown) in agreement with Andersson *et al.*²³ who pretreated cells with anti-Ig at 4 °C before culturing with LPS. The anti-Ig beads, however, did not turn off Ig secretion in cultures stimulated with LPS (Table 1). (2) T cells or macrophages may be inhibiting Ig secretion. (3) The anti-Ig beads may be giving a proliferation signal to a subpopulation²⁴ of B cells which requires a second differentiation signal to proceed to a high rate of Ig synthesis and secretion^{25,26}. Soluble anti-Ig stimulates

proliferation of rabbit lymphocytes by cross linking receptors, but antibody secretion depends on the addition of T-cell factors²⁷. This radical dissociation of proliferation from immunoglobulin secretion in anti-Ig bead-stimulated B cells will prove useful as a model for regulation of antibody synthesis in antigen-activated B cells in the mouse, where the dissection of interacting components in the immune response has proceeded much further than in other species.

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Identical sequence of light chains from rabbit anti-streptococcal antibodies

CONSIDERABLE advances have been made with special breeding programmes in the selection of rabbits with amplified immune responses to polysaccharides specific for different streptococcal groups^{1,2}. Levels of group specific antibodies of 50 mg ml⁻¹

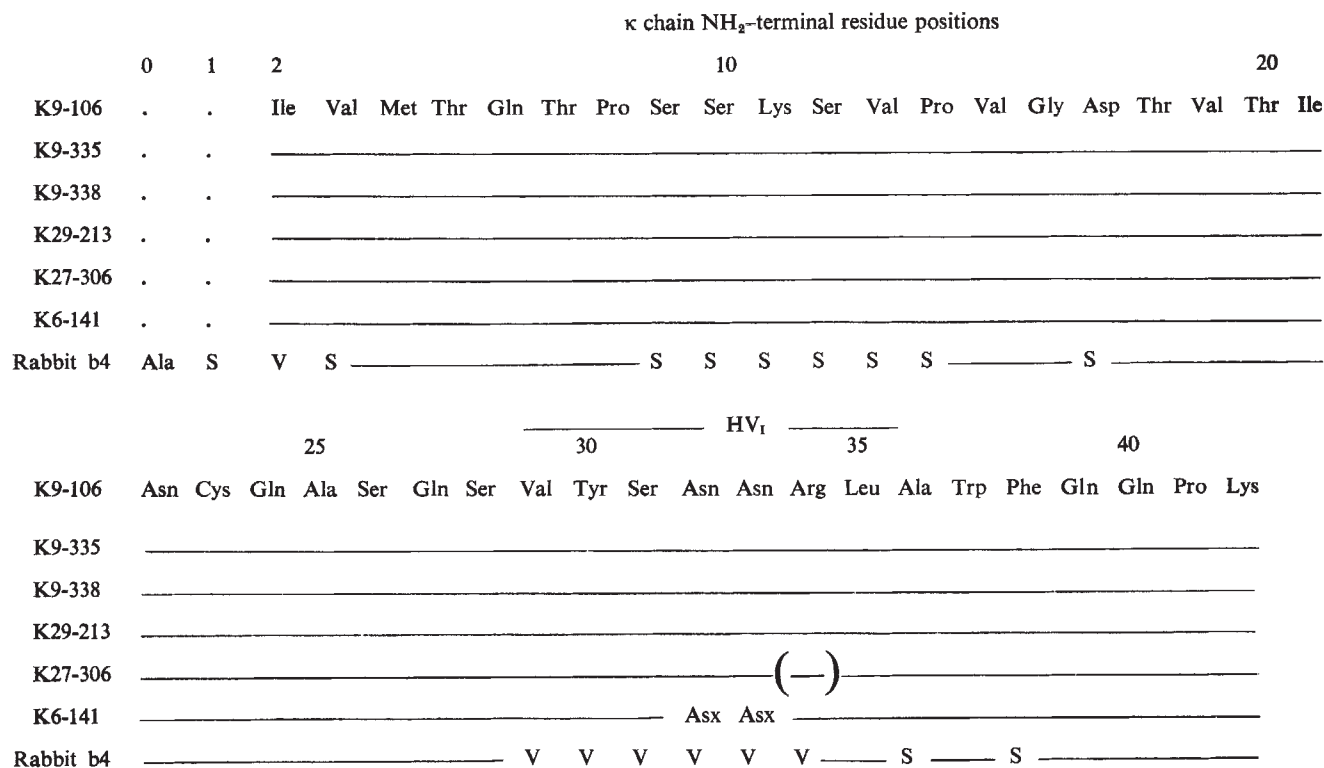


Fig.1 The amino-terminal sequences of rabbit allotype b4k light chains isolated from antibodies to the streptococcal polysaccharide. Solid lines indicate sequence identity to the leading sequence. Asx, undetermined asparagine residue. HV₁ marks the first hypervariable region¹⁴. For comparison a prototype rabbit b4 sequence is indicated². S indicates positions that can express subgroup specific residues; V indicates highly variable positions. Dots mark deletions.

have been reported. Such antisera are valuable because they often contain monoclonal antibody which can be used in studies of the origin of antibody diversity. These antibody probes have stimulated a search for variable region markers common to antibodies specific for the same polysaccharide antigen³ and produced by closely related rabbits^{4,7}. In a continuation of earlier marker analyses³ and of a breeding programme for restricted high responders⁸, we have used the streptococcal group A variant antigen (strain A486 variant of the Rockefeller University) to immunise part-inbred rabbits^{6,7}. This cell wall polysaccharide is a homopolymer of rhamnose^{9,10} of very limited heterogeneity. We report the partial amino acid sequences of light chains of the b4 allotype isolated from monoclonal antibodies raised in six closely related rabbits after a primary or secondary course of intravenous immunisation with a streptococcal group A variant vaccine⁶.

The light chains were selected for their amino-terminal isoleucine residue. Previous work had shown that such light chains belong to a separate subgroup which accounts for no more than 3% in a rabbit immunoglobulin pool^{11,12}.

Rabbits K9-106, K9-335 and K9-338 were littermates from a mating of unrelated parents (K429-1♂ × K23♀), rabbit K6-141 arose from a brother-sister mating of K19♀ × K20♂, which were littermates to K23; rabbits K29-213 and K27-306 were progeny of K9-106 with its siblings K9-107 and K9-133.

Partial amino acid sequence analyses were performed with a Beckman 890B sequencer. Both partially reduced and alkylated light chains and fully reduced and alkylated light chains were used. Fully reduced samples were alkylated with ^{14}C -labelled iodoacetic acid (Radiochemical Centre, Amersham). The quadrol program was used for all analyses. All phenylthiohydantoin amino acid derivatives were analysed by gas chromatography (Beckman GC45 gas chromatograph)¹², by thin-layer chromatography on silica gels with identification by the starch-iodine method¹³, and by amino acid analyses after back-hydrolysis with hydriodo acid to free amino acids¹².

Figure 1 shows the sequences of the six light chains up to

position 42. They include framework residues (positions 2-28 and 36-42) and the entire first hypervariable region (positions 29-35)¹⁴. The principal conclusion is the identity in sequence from positions 2-42. In contrast, the hypervariable regions from two other light chains isolated from two anti-azobenzene arsonate antibody fractions produced by one rabbit¹⁵ differed at positions 30-34, although they belonged to the same subgroup. Also light chains of separate subgroups that were isolated from antibodies to *p*-azobenzoate¹⁶, pneumococcal type III polysaccharide¹⁷ and streptococcal group C polysaccharide¹⁸ differed significantly in the first hypervariable region. It is therefore likely that the unique sequence of the first hypervariable region of these light chains correlates with the antigen specificity. Light chains of this subgroup were found previously in streptococcal group C specific antibodies¹¹. The amino acid sequences of their first hypervariable regions are not yet available.

Valine at position 11 and glycine at position 17 were designated species-specific for rabbit κ chains of the b4 allotype because no alternative amino acids were found in these positions in rabbit light chain sequences available at that time¹¹. As such, they served in arguments about the diversification of rabbit light chains as the basis for a proposed gene expansion and contraction mechanism^{11,19}. But, the finding of lysine at position 11 and aspartic acid at position 17 in six rabbit light chains from allotype b4 rabbits shows that these positions are not occupied by species-specific amino acid residues that could serve as markers. Rather, it is likely that they mark subgroup-specific positions characteristic for the amino terminal sequence Ile-Val-Met. None of the light chains was associated with a particular heavy chain allotype; they occurred either with heavy chains of the group *a2* marker (K9-106, K9-335, K9-338 and K29-213) or with the group *a3* marker (K27-306 and K6-141). Accordingly they were found in antibodies of variable and overlapping isoelectric points.

All but light chain K6-141 were associated with dominant clonotypes. The dominant clonotype of rabbit K6-141 anti-

A-variant polysaccharide antibodies at the peak of the antibody response was characterised by an amino-terminal sequence of Ala-Leu-Val. This sequence is characteristic of another subgroup of rabbit antibody light chains^{11,12}.

Barstad *et al.* showed identical amino-terminal sequences of three light and five heavy immunoglobulin chains from myeloma proteins of BALB/c mice with binding activity to phosphorylcholine²⁰. Our finding of sequence identity from residues 1–41 in light chains produced by closely related but not inbred rabbits, in response to streptococcal A variant polysaccharide suggests that these variable light chain regions are the products of a germ line gene, and not generated by random somatic hypermutation and subsequent selection. A fundamental insight into the selection of antibody light chains to streptococcal polysaccharide antigens which share a rhamnose backbone^{1,9} can be expected from the sequence analysis of light chains derived from anti-streptococcal group C antibodies with the amino-terminal sequence Ile-Val-Met¹¹.

Our data also suggest that single rabbits (for example, K6-141) can respond to the streptococcal group A variant polysaccharide with antibodies containing light chains of two separate subgroups. This is not consistent with the view that a basic nucleotide sequence serves as a germ-line gene-encoded matrix from which new structural genes are derived by subsequent step mutations. It rather suggests two separate germ line genes for one antigen.

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Syntheses and immunological evaluation of bovine proinsulin C-peptide analogues

PROINSULIN is a single-chain polypeptide in which the A and B chains of insulin are linked together by the connecting peptide¹. The terminals of the connecting peptides are occupied by the basic dipeptides, Arg-Arg and Lys-Arg, respectively, and proinsulin C-peptide is defined as connecting peptide minus the basic dipeptides. The immunological reactivity of synthetic [59-formyllysine]-bovine proinsulin 31–60, which comprises the entire sequence of the connecting peptide segment of bovine proinsulin, is indistinguishable from that of natural bovine proinsulin when compared on an equimolar basis². The immunoassay system involved a bovine proinsulin antiserum³, which was purified by the removal of the antibodies directed towards the insulin region of the molecule. We have now evaluated the structural features of bovine connecting peptide segment which determine its reactivity with bovine proinsulin antisera. For

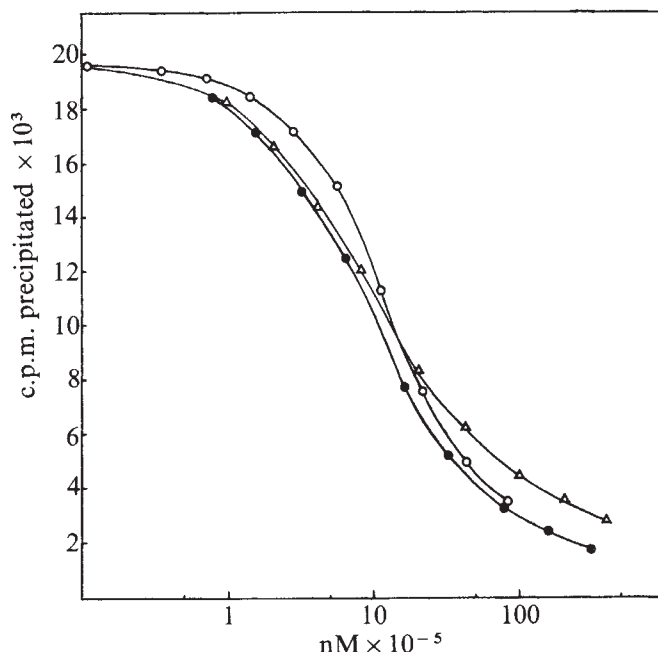


Fig. 1 Double-antibody radioimmunoassay using bovine proinsulin antiserum and ¹²⁵I-tyrosylated [59-formyllysine]-bovine proinsulin 31–60 as tracer. ●, Synthetic [59-formyllysine]-bovine proinsulin 31–60; △, natural bovine C-peptide; ○, natural bovine proinsulin.

this purpose, homogeneous synthetic peptides of defined structures serve as excellent substrates. We synthesised twelve peptides related to bovine proinsulin C-peptide which has the following amino acid sequence

33
Glu-Val-Glu-Gly-Pro-Gln-Val-Gly-Ala-Leu-Glu-Leu-Ala-
58
Gly-Gly-Pro-Gly-Ala-Gly-Gly-Leu-Glu-Gly-Pro-Pro-Gln

The peptides were constructed entirely by the fragment condensation method in solution using catalytic hydrogenolysis to remove the benzyloxycarbonyl group as amino protection. Mixed anhydrides⁴ or azides⁵ of protected di-, tri- and tetrapeptides were used for acylation. The final products were purified exclusively by CM-Sephadex (free form) column chromatography using increasing concentrations of acetic acid for elution. Each of the ensuing products was homogeneous as judged by thin-layer chromatography, and acid hydrolysates of these peptides contained the constituent amino acids in the ratios predicted by theory. The synthetic bovine C-peptide fragments were as follows: bovine proinsulin (BP) 33–46, BP 33–50, BP 33–53, BP 33–54, BP 37–46, BP 37–49, BP 37–50, BP 37–52, BP 37–53, BP 39–52, BP 41–53 and BP 41–50.

Immunological reactivities of these synthetic peptides were determined by the double antibody method³ using an antiserum against natural bovine proinsulin raised in a rabbit and ¹²⁵I-synthetic tyrosylated [59-formyllysine]-bovine proinsulin 31–60 as tracer. The tyrosylated derivative was obtained by the interaction of benzyloxycarbonyltyrosine N-hydroxy-succinimido ester⁶ with [59-formyllysine]-bovine proinsulin 31–60 followed by hydrogenolysis. This peptide was iodinated by a modification of the method of Frechet *et al.*⁷.

Figure 1 shows the relative immunoreactivity of natural bovine proinsulin and C-peptide and synthetic [59-formyllysine]-bovine proinsulin 31–60. The result indicates that the synthetic peptide reacts similarly to endogenous bovine proinsulin and C-peptide on an equimolar basis.

The displacement curves of the synthetic bovine proinsulin C-peptide fragments are shown in Fig. 2. All the peptides

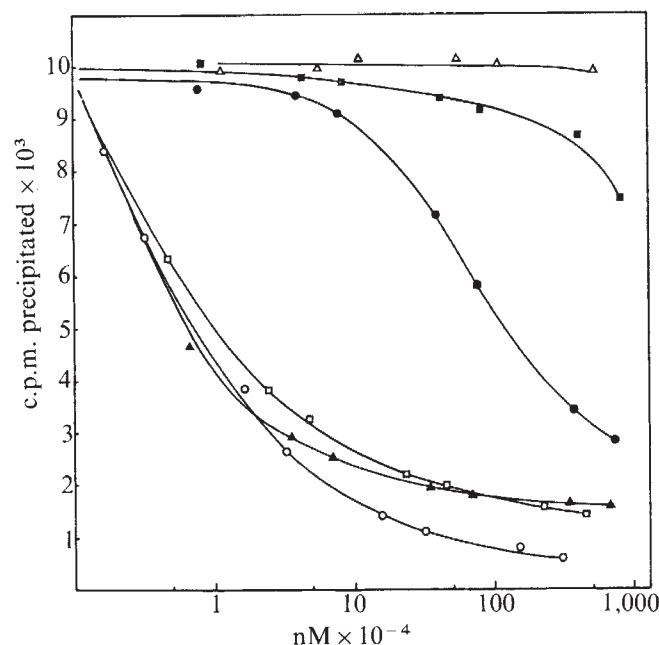
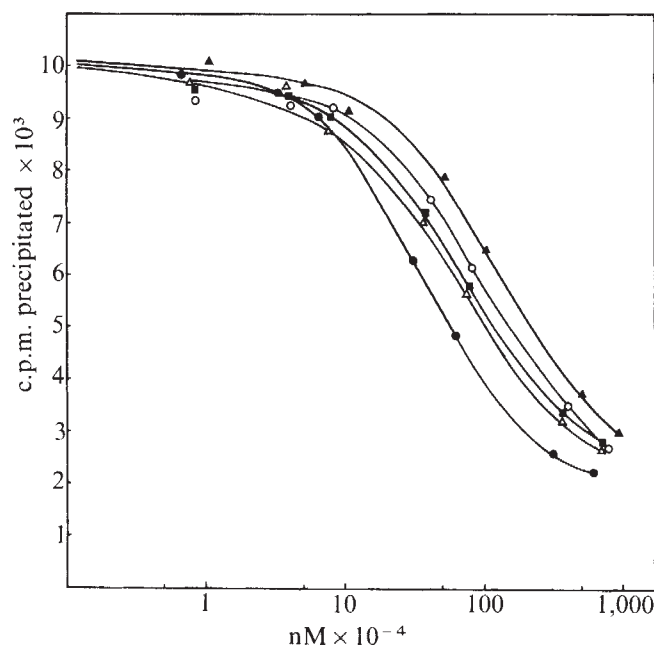


Fig. 2 Double-antibody radioimmunoassay of synthetic peptides related to bovine proinsulin C-peptide using bovine proinsulin antiserum. 125 I-tyrosylated [59-formyllysine]-bovine proinsulin 31-60 was used as tracer. ○, [59-Formyllysine]-bovine proinsulin 31-60; ▲, BP 33-46; □, BP 33-50, BP 33-53, and BP 33-54; ●, BP 37-46, BP 37-49, BP 37-50, BP 37-52, and BP 37-53; ■, BP 39-52; △, BP 41-50 and BP 41-53.

that contained the amino acid sequence corresponding to the bovine proinsulin 33-46 fragment were found to cross react almost identically with synthetic [59-formyllysine]-bovine proinsulin 31-60. Displacement curves of BP 33-50, BP 33-53 and BP 33-54 were plotted as one representative line because of their similar reactivities. BP 37-46, BP 37-49, BP 37-50, BP 37-52 and BP 37-53 displaced the tracer similarly, as shown by the mean of their curves. These peptides, however, exhibited significantly reduced cross reactivity. The shorter peptides, BP 39-53, still reacted though very weakly, whereas neither

Fig. 3 Competition between BP 37-46 (▲), BP 37-49 (○), BP 37-50 (■), BP 37-52 (△), and BP 37-53 (●) and 125 I-tyrosylated [59-formyllysine]-bovine proinsulin 31-60 for binding to bovine proinsulin antiserum.



BP 41-50 nor BP 41-53 competed with the tracer at the concentrations we used. The marked difference of the cross reactivities of BP 33-53, BP 37-52, BP 39-52 and BP 41-53 clearly indicates the effect of decreasing the chain length in that part of the molecule which contributes to the interaction with its antibody. Our investigation shows that the major antigenic region in the connecting peptide portion of bovine proinsulin is located within residues 33-46 in the amino acid sequence. The evidence was obtained with the major anti-C-peptide antibody but certainly not the only one in the antiserum studied. Removal of four amino acid residues, Glu-Val-Glu-Gly, resulted in a marked decrease in immunoreactivity. The proline residue at position 37 seems to be of special importance. Peptides, BP 39-52, BP 41-50 and BP 41-53 which do not contain the proline residue at position 37 show extremely low reactivity or do not react at all. Figure 3 represents individual displacement curves of BP 37-46, BP 37-49, BP 37-50, BP 37-52 and BP 37-53. There is a gradation of increasing reactivity with increase of the chain length from the carboxyl end of BP 37-46. A partial contribution of the 47-60 sequence on the immunological reactivity has been suggested from our observation² that an inversion of residues 50 and 52 in the bovine proinsulin connecting peptide segment resulted in a distinct loss of immunoreactivity. The observations reported here also indicate that the 46-53 sequence participates in binding, although this contribution is rather small.

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Errata

In the article "Scotopic and photopic dark adaptation of the b wave in isolated rat retina" by W. Ernst and C. M. Kemp (*Nature*, **258**, 170; 1975) the sentence that begins in line 17 of the second paragraph should read ... This means that either bleaching has caused some permanent change in the way light of different wavelengths reaches the rhodopsin molecules, or the b-wave cells are controlled by a non-rhodopsin system. ... and not as printed.

The correct authorship of the article "Synthesis mimics of insect juvenile hormone" (*Nature*, **232**, 486; 1971) should be

Ferenc M. Pallos
Hwalin Lee
Peter E. Letchworth
John B. Miaullis
Julius J. Menn

and not as printed in the original article or in the corrigendum which appeared in *Nature* (**253**, 288; 1975).

reviews

In 1967 a group of geographers in the US and Canada initiated a study of the human and social responses to natural hazards in different parts of the world. The sort of questions they hoped to answer were: How many people inhabit particular areas of hazard and why do they continue to do so? How do these inhabitants perceive the extreme natural events to which they are subject? What options are open to them, both individually and collectively, in adjusting to the dangers and how are these options exercised? For six years, investigators in more than 23 countries collaborated in interviewing samples of populations at risk from earthquakes in San Francisco, cyclones in Bangladesh, drought in Kenya and Australia, floods in India and Britain, volcanic eruptions in Hawaii, avalanches in Norway, and various other hazards in nations at different stages of development.

The results of this 'loosely coordinated' survey, many of which are described in the 32 articles in *Natural Hazards*, are disturbing, although perhaps not entirely surprising. Galveston, Texas, is prone to hurricanes; but although most residents of the city are aware of the threat, about a third of them refuse to believe they will ever be affected personally and therefore take no precautions. About a fifth of San Franciscans think they will never experience earthquake damage. Over half of those who live on the River Severn floodplain in Shrewsbury, England, not only do not expect to witness a flood in the future but do not even know they have a flood problem. The list goes on and on; and it all makes fascinating reading.

The point of this study, however, is

Hazards of life

Natural Hazards: Local, National, Global. Edited by Gilbert F. White. Pp. xvi+288. (Oxford University: New York, London and Toronto, 1975.) £7.50. *Geological Hazards: Earthquakes - Tsunamis - Volcanoes - Avalanches - Landslides - Floods.* By B. A. Bolt, W. L. Horn, G. A. Macdonald and R. F. Scott. Pp. viii+328. (Springer: Berlin and New York, 1975.) *Physical Aspects of Natural Catastrophes.* By Adrian E. Scheidegger. Pp. xiii+289. (Elsevier: Amsterdam, Oxford and New York, 1975). Dfl. 70; \$29.25.

not to catalogue human ignorance and irrationality for their own sake but to assess the nature of the real responses with which public policy to mitigate the effects of natural hazards must contend. All too often in the past, technological solutions to the problem of coping with hazards have failed because they were based on cost-benefit analyses which assumed that people will always behave in an economically rational way. The cost of flood damage in the US, for example, has continued to increase alarmingly in spite of a multi-billion dollar investment in flood control works spread over more than 20 years. Likewise, hurricane warning systems, however accurate, have been less than completely effective in convincing the public to take avoidance measures. The fact is that real people, given freedom of choice, seldom behave in the tidy way that policy makers would wish. This may be partly through ignorance and partly through misguided optimism, but it also reflects the existence of

more than one scale of human values.

The importance of *Natural Hazards* is that it gives substance in a particular context to the old allegation that there is nothing so unpredictable as people and points up the folly of those who have persistently ignored this simple fact of life. The book should therefore be essential reading for all those scientists who see complete solutions to the problems of natural hazards in the rational pursuit of scientific knowledge and to all those politicians and administrators who have been misled into believing that all physical and human nature may ultimately be subdued by the technological fix.

But although the scientific approach may not be sufficient to cope with natural hazards, it is certainly necessary, and is given due prominence in *Geological Hazards*. Presumably the original work of Gilbert F. White and his colleagues is still too new to have reached the secondary texts, and so there is little here about the vagaries of human behaviour. On the other hand, although primarily a book about science, the science is placed firmly in the social context which gives it point. As a general (descriptive and non-mathematical) introduction to a wide range of natural hazards, this book may be recommended without reserve.

With neither the common theme of human response nor the self-justifying interest of the general reader, the linking of different hazards in *Physical Aspects of Natural Catastrophes* has less point. Intended largely as a scientific source book for engineers, the book is mathematical and utilitarian. It may be referred to, if necessary, but is unlikely to be read.

Peter J. Smith

THE first chapter is devoted to the methods and results of the LCAO-MO Hückel approach. Though the first section of this chapter is headed "Orbitals", I feel that a reader of this book would need to know something about atomic and molecular quantum mechanics before he started. It seemed to me surprising to devote so much of the first chapter of a book like this to π -orbitals, though I think the author is very sensible to use the allyl system as his example.

In the second chapter Zimmerman deals with eigenvalues, eigenfunctions operators and the variation method, which is dealt with clearly at some

Quantum mechanics

Quantum Mechanics for Organic Chemists. By Howard E. Zimmerman. Pp. x+215. (Academic: New York and London, May 1975.) \$16.50; £7.90.

length. The next chapter explains the effects of molecular symmetry and introduces the reader to group theory. After that the book returns to the Hückel method and extends the earlier consideration, continuing with an account of more advanced treatments. Systems are treated as containing many interacting electrons; Slater determinants and the SCF method are

described. There is also a description of configuration interaction and examples of its applications.

It is fair to say that this book approaches and tackles the problem of presenting quantum mechanics to organic chemists in a manner different from that used by other books. As such, it is interesting and stimulating; I certainly found it very much worth studying. But I must say also that I was not left convinced that the treatment and sequence used is the best way to tackle the job; but it deserves consideration and parts are well done and most useful.

J. W. Linnett

Giant algal cells

The Physiology of Giant Algal Cells. By A. B. Hope and N. A. Walker. Pp. xiii+201. (Cambridge University Press: London, 1975.) £8.50; \$24.95.

THIS small book, on what may at first seem a restricted subject, blossoms to cover a range of electrophysiological phenomena in both animals and plants. The plants concerned (mainly Charophyta, with mention of appropriate Bryopsidophyceae, Chlorococcales and Ceramiales), and also the basic electrophysiological techniques involved, are adequately introduced in the first chapter. The glossy photographs of *Chara*, *Nitella* and *Griffithsia* could, however, have been replaced by more electronmicrographs.

Chapter 2 provides a chronology of work from 1774 up to 1938, but the subsequent period of consolidation of earlier ideas is what this book is really about. Chapter 3 starts off gently with "Water Relations", although the biophysical leanings of the book are already apparent in the extensive mathematical treatments which place perhaps too much dependence on hieroglyphics (51 special symbols and 32 abbreviations are listed at the front) and which make too little attempt to put important concepts clearly in words, a characteristic of the rest of the book.

In the following chapters little consideration is given to at least one part

of the professed readership: undergraduate students; and recommendation of this book to the majority of plant physiology students would lead to nothing short of baffling disaster. The specialist, on the other hand, may be repeatedly frustrated at being sent off to the literature because of the lack of detail in the rather brief text. It is difficult to determine whether the book is meant to be a thorough review article on a very wide-based topic or a half-hearted textbook of plant electrochemistry; the authors have perhaps not succeeded in striking an adequate balance between the two approaches and have obviously been hampered by lack of space. Some topics receive unfortunately little coverage: photosynthesis, for instance, is only mentioned as an adjunct to several other processes which are affected by light, such as cation uptake, and carbon fixation rates are given minimum space.

The final chapter is refreshingly different, providing a delightfully empathised account of the beauty and mechanism of protoplasmic streaming, the earliest recorded phenomenon in these giant cells and, apparently, the least quantified. Unfortunately, it does nothing to draw to a conclusion the mass of data in previous pages. The lack of such a concluding section is especially serious in a book so full of extremely critical comment on everyone's efforts, including the authors' own researches. **Edward A. Drew**

Drug resistance

Infectious Multiple Drug Resistance. By S. Falkow. Pp. 300. (Pion: London, July 1975.) \$19.95; £7.70.

THIS is a very useful book which is in general up to date. It is the only treatise so far published which exposes, with considerable success, the practical as well as the theoretical significance of bacterial plasmids. It is a pity that the title is misleading: not only does the book deal with infectious (I prefer "transferable") drug resistance, but it presents the reader with a succinct introduction to microbial genetics and also contains a substantial amount of the relevant molecular biology. Moreover, it describes in some detail the plasmids that may contribute to virulence, at least in the potentially pathogenic *Escherichia coli* of animals and probably of man.

There is something here for everyone interested in the field: fundamental information about the structure of bacterial transfer systems; clinical data about the incidence of bacteria carrying R factors and other plasmids in man and animals; information about and speculation on the origin of the resistance and other genetic determinants and the transfer factors that can carry them, and the relationship between the two types of agent in complete transfer systems; and a useful discussion of the ecological significance of plasmid-determined resistance.

There is, however, no specific identification of references in the text. In a book of this stature, all references should be precisely indicated in the text, so that they can be related to the full references at the end of the chapter, or of the book. Chapter 4 on ecology finds itself in the genetic introduction, instead of in conjunction with chapter 10, which deals with *in vivo* transfer of R factors and their significance in public health. The terms f^+ and f^- are uncomfortably near synonymy with F-like and I-like throughout the book. And there was sufficient information available about the Mexican strain of chloramphenicol-resistant *Salmonella typhi* and the R factor it carried, while this book was being written, to warrant fuller description of this highly important example of the disasters that may follow the abuse of antibacterial drugs. The designation of compatibility Group O is given undeserved priority. Group B, which is identical with it, was described over a year before O.

But I am carping, and I am sure the next edition will rectify the small scattered faults. The literary style is rather breezy—not necessarily a disadvantage—and I would strongly recommend this book. **E. S. Anderson**

Special relativity

Special Relativity. (Oxford Physics Series.) By J. G. Taylor. Pp. 106. (Clarendon: Oxford, Oxford University: London, June 1975.) £1.95.

FOR the past three years Professor Taylor has been giving a series of lectures on introductory special relativity to first-year undergraduates at King's College London. The content and spirit of these lectures now appears in a compact and very readable book from the Oxford Physics Series. The approach is a modern mathematical one, but physics students will find little difficulty in following the text. The first two chapters deal with the physical basis of special relativity, such as space and time measurements. The author deliberately adopts the language and concepts of relativity theory at the outset, rather than the traditional method of building them on an erroneous foundation of Newtonian physics. In this way the theory emerges in a very natural and elegant fashion. Starting solely from the constancy of

the speed of light, the Lorentz transformation is introduced. The formal treatment which follows is based on the structure of the Lorentz group, and deals skilfully and concisely with all the major areas of interest in relativistic kinematics and dynamics. Students with a knowledge of vector field theory and electromagnetism will benefit most from this particular approach, although this book will prove a useful teaching text in almost any mathematics or physics department.

A final chapter is included on extensions to special relativity. Tachyons (hypothetical particles which travel faster than light) are discussed in unusual depth for a course book, and the question of their existence left open. The reader is then taken to the beginnings of the general theory of relativity with a qualitative account of acceleration effects and the equivalence principle. Some problems and their solutions are given at the end.

In summary, Professor Taylor has produced an uncomplicated, economical and informative account of the special theory of relativity in an elegant modern style. **P. C. W. Davies**

Environmental physiology

Environmental Physiology. By D. Bellamy, G. J. Goldsworthy, K. C. Highnam, W. Mordue and J. G. Phillips (Editor). Pp. ix+198. (Blackwell Scientific: Oxford and London, 1975.) £3.25.

THIS book attempts to present, at a fairly introductory level, a general view of physiology as it relates to animals in their natural environment; as is stated in the preface, it seeks to give expression to the conceptual link between physiology and ecology. The text is divided into three parts. The first of these introduces the animal and its environment, pointing out the main relationships between them, and goes on to consider the internal environment of the organism. As with any introductory text, basic concepts need to be outlined and it is in this first part of the book that the physiological ideas of homeostasis, active transport, osmotic pressure, nerve impulses and the like are explained.

The orientation of the material presented in the second and third parts is more ecological. Part two examines the problems and mechanisms which are associated with the organism-environment interfaces. The structure and function of the epidermis, gills and lungs, excretory organs and the alimentary tract are

treated phyletically. These chapters are liberally sprinkled with examples which bring out the underlying problems facing the chosen animal types in different habitats. The third part of the book deals with specific interactions of organisms with their environment, beginning with what might at first seem to be a curious chapter to have in an essentially physiological text. The chapter is entitled 'Global diversity and animal distribution' and explains how and why climate and season vary over the earth's surface. A useful table is presented which summarises the physical features of the main biogeographical areas, their characteristic plants and animals, and the adaptations enabling these organisms to exist in each area. This chapter proves to be a valuable introduction to the third part. A series of specific problems facing animals in certain environments are discussed, with particular stress on salt and water availability, and there is also a chapter on the maintenance and function of rhythms. This is followed by a complementary chapter on the effect of seasonal rhythms on the organisms, with particular emphasis on diapause.

The third part ends with three short chapters, one on animal colour and colour change, one on biotic relationships between organisms, and a final chapter on man's impact on the environment. The biotic relationships chapter really only whets the appetite for the increasingly important field of behaviour, and much the same sort of comment can be applied to the last chapter in which the topic of pollution is scarcely introduced. In a book of this title rather larger components of behaviour and pollution might be expected, particularly as regards the physiological effects of man's pollutants on organisms.

The general finish of the book is high although the plastic laminate of the paperback version tends to part company easily from the rest of the cover, and the book quickly becomes dog-eared in use. The overall impression is that it is a useful first and second year reading for undergraduates in both physiology and ecology and that this book may also find a place in sixth form teaching.

Bryan D. Turner

Practically everyone who is anyone in the field of cellular immunology seems to have been there, searching for answers to many of the outstanding questions about the control and expression of immune responses at the molecular level.

Although there were a few reports on the structural basis of antigen (or haptan) binding sites, particularly in relation to antibody specificity and diversity, and even half a paper on the IgE system, the main emphasis was placed firmly on the genetic and cellular control of antibody synthesis.

The reader of the proceedings unfortunately misses what (from the Preface) must have been much lively and uninhibited discussion at the Workshop sessions; transcripts of which have not been included. But the published papers cover a wide range of 'up-to-the-minute' topics in the vanguard areas of immunology and should, therefore, provide informative reading for workers in many different branches of the discipline.

Inevitably many questions are left unanswered, including some key ones—such as the nature of T cell surface receptor. Nevertheless, the reader is treated to stimulating new thoughts about fascinating topics like the nature and control of antibody diversity, the relationship between the genetic control of histocompatibility antigens and immune responses, lymphocyte membrane organisation, the mechanism of development of B memory cells, and the role of T cells in immune responsiveness and tolerance. The proceedings also contain a range of valuable technical information, including descriptions of: the 'finger printing' of thymus cells by physicochemical procedures, the fractionation of lymphocytes and probing of their surface structure, the estimation of messengerRNA responsible for Ig heavy chain synthesis and the measurement of clonal development of single antibody-forming cells in culture.

The only disappointing aspect of the book is that dealing with the triggering of lymphoid cells. The treatment of mechanistic aspects of cell activation and transformation is sparse to say the least, in spite of the reference to 'receptors' and 'signals' in the title. This no doubt reflects the wide gap that still exists between immunological observation and biochemical understanding of lymphocyte stimulation processes. In view of the growing evidence, however, that immunological triggering processes have important features in common irrespective of the nature of the target cell, it is to be hoped that the next Symposium on this topic will take into consideration the valuable clues about lymphocyte function to be gleaned from the fundamental investigations now in progress on other immunologically controlled cell systems such as those involving mast cells and macrophages. Denis R. Stanworth

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Genes, receptors, signals

The Immune System: Genes, Receptors, Signals. Edited by E. W. Sercarz, A. R. Williamson, and C. F. Fox. Pp. xxiv+632. (Academic Press, New York and London, 1974.) \$22.50.

It becomes increasingly obvious on reading the proceedings of the Third ICN-UCLA Symposium on Molecular Biology that an immunological event of some importance took place at Squaw Valley, California, in March of last year.

Flavonoids

The Flavonoids. By J. B. Harborne, T. J. Mabry and H. Mabry. Pp. 1,204. (Chapman and Hall: London, July 1975.) £27.50.

THE appearance of *Chemistry of the Flavonoid Compounds* edited by Geissman in 1962 and of *Naturally Occurring Oxygen Ring Compounds* by Dean in 1963 were major publishing events in the flavonoid field, and undoubtedly did much to stimulate the impressive advances which have taken place since their publication, over a decade ago. The present volume, edited by active contributors to the subject, attempts to summarise progress in the flavonoid field since Geissman's book. The former work will, however, still deserve a place on the library shelf for reference to some of the older work.

The chapters of *The Flavonoids* fall into three main groups. Firstly, four chapters concerned with isolation techniques, ultraviolet and nuclear magnetic resonance spectroscopy, mass spectroscopy and synthetic methods. Secondly, eleven chapters concerned with the individual classes of flavonoids. Finally there are five interrelated chapters on biosynthesis, metabolism, physiology and functions, biochemical systematics, evolution.

This is a work of major importance; the many phytochemists who have been eagerly awaiting its publication will not be disappointed. It is inevitable that there will be the odd error, misquotation or missed reference; the reviewer found some pertaining to the Rosaceae, and doubtless others will find likewise within their own specialities. Overall, the editors could have exercised a greater degree of control over the general structure of each author's contribution; for example, although there may be justification for treating flavone and flavonol aglycones in separate chapters, it would be reasonable to expect the separate treatments to be along closely parallel lines—which they are not. In both, there is unnecessary overlap with material contained in the first four chapters. It seems strange that chalcones and flavanones should be dealt with in separate chapters—a somewhat unsatisfactory arrangement.

Coverage of the literature is uneven; some contributors have reviewed their topics from their beginnings, others have taken Geissman's 1962 book as their starting point and the chapter on anthocyanins takes Harborne's 1967 *Comparative Biochemistry of the Flavonoids* as its starting point in listing natural occurrences. It is not always easy to ascertain in which year the literature coverage stops, as in some chapters it is extended by an addendum

at the end of the book; it does seem, however, to be intensive up to the end of 1972, with somewhat patchy and incomplete coverage of the 1973–74 literature (appearing often in both main text and addendum). Such inconsistencies are, however, difficult to avoid in such multi-author works.

The first fifteen chapters are highly factual in content, as obviously intended, but nevertheless frequently make interesting reading. The last five chapters, however, because of their more general nature, make more enjoyable, enlightening and occasionally provocative reading. One notable omission from the chapter on biosynthesis is any mention of the possible rôle of the interrelated α - and β -hydroxychalcones, chalcone epoxides, 2-hydroxyflavanones and dibenzoyl-methanes in flavonoid biosynthesis; these compounds have recently aroused interest as possible biosynthetic intermediates and some informed speculations would have been welcome.

Throughout the remaining chapters there is a steadfast refusal even to admit the possibility that flavonoids (with the obvious exception of flower pigments) might be generally functionless. Suggested functions range from light screens to sex determination. Many supposed physiological functions of flavonoids might be the consequence, rather than the cause, of changes in primary metabolism. If in fact flavonoids are generally functionless, then the phylogenetic consequences are of considerable interest; this aspect has not even been considered.

The concept of 'replacement characters,' referred to a number of times (for example, page 1,066) seems of dubious validity to the reviewer. Why should betacyanin pigments replace anthocyanins, or dihydrochalcones replace arbutin? Flavones are said to replace flavonols (page 1,067) yet it would seem more reasonable to say that flavones seem in some instances to co-exist with the more common flavonols, and in some instances flavones occur alone. In the reviewer's experience, when flavones appear in certain *Pyrus* species, the flavonol levels can actually be much higher than otherwise; how can this be defined as a replacement?

Criticisms apart, the book is undoubtedly a major publishing event in the field of phytochemistry. The general layout is tidy, spacious and uncluttered, and the indexes (authors, plant species and subjects) are excellent. *The Flavonoids* will serve as a major and indispensable work of reference for many years to come and can be unreservedly recommended to anyone concerned with this subject. Unfortunately the price will restrict this book to few purchasers other than libraries.

J. S. Challice

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December 1975: 64 pages: illustrated: 412 13910 3: paperback: £1.30.

Cytogenetics is primarily concerned with the structure and function of chromosomes. This book provides an up-to-date account of recent techniques and results in this field laying particular stress on abnormalities and their medical relevance.

Molecular Virology

T. H. PENNINGTON AND D. A. RITCHIE

December 1975: 64 pages: illustrated: 412 12590 0: paperback: £1.30.

In this introductory account, the authors discuss the fundamental aspects of virology at the cellular and sub-cellular levels. Their approach to the subject is balanced and equal coverage is extended to animal viruses and bacteriophages.

CHAPMAN & HALL
11 New Fetter Lane, London EC4P 4EE



obituary

Conrad Hal Waddington, CBE, FRS, the noted geneticist and distinguished scientific thinker, died on September 26 at the age of 69.

HAVING graduated from Cambridge (Sidney Sussex) in 1926 with a first class honours in geology, Professor Waddington became lecturer in zoology at Strangeways Research Laboratory, Cambridge in 1933, but later was persuaded to move to embryology in 1936. In 1947, two years after leaving Strangeways, he became Buchanan Professor of Animal Genetics in the University of Edinburgh, where he remained until his death. His practical involvements in international affairs include the presidency of the International Union of Biological Sciences from 1961–67 and the setting up and vice-presidency of the International Biological Programme (1963–66). He was also a founder member of the Pugwash Conference and the Club of Rome. His most outstanding contributions were, however, as a synthesiser of knowledge and as a populariser of science through his many books and papers. His many honours include FRS (1947), CBE (1958), foreign membership of the American Academy of Arts and Sciences, several honorary doctorates and numerous visiting lectureships and fellowships.

THE bare, formal, record of Waddington's career gives little indication that he differed much from other distinguished scientists of his generation. Yet Waddington was a very unusual man, and if his influence on science was diffuse—he established no defined school as a legacy—it was certainly very widely felt.

The transition from geology through evolutionary studies to genetics was natural, but thwarted at first by lack of employment in genetics. Experimental embryology was the next best thing. Alone, and in collaboration with the Needhams and with Jean Brachet, his ten years in Cambridge saw a number of important contributions to our knowledge of embryonic induction. Most significant were the establishment of the phenomenon in higher vertebrates, a technically formidable undertaking, and, in the second place, the discovery of inductive activity in a chemically defined but unnatural substance (methylene blue). At the same time he was theoretically active in refining the concepts of induction and,

more generally, in relating the mechanics of development to the expression of genes. Many of his views (for example, in *Organisers and Genes*, 1940) are recognisable in today's thinking.

It was to be expected that he should move onto the developmental genetics of *Drosophila*, but this also heralded the beginning of a pattern that was to persist. His own experimental efforts became eclectic. He could not resist a good idea for an experiment, but he, or his students, picked up too many interesting problems for him to call any one field his own. The positive side of this breadth of sympathy was real



enough. The Institute of Animal Genetics in Edinburgh became justly celebrated for work in a quite exceptional range of genetic and developmental fields. Waddington provided an umbrella under which important talents could shelter.

His own science became centred around the problems of relating genetics to adaptive evolutionary change by way of developmental processes. Waddington grappled with the constraints that developmental considerations place on the kinds of evolutionary change that are practicable in a succession of books (for example, *Strategy of the Genes*, 1957) and articles of great interest. A brief account of his scientific life (in *Towards a Theoretical Biology*, Volume 2, 1969), illustrates the enduring influence which A. N. Whitehead had on

his mind, and that he was first and foremost an evolutionary biologist for whom the mechanisms of development and inheritance revealed by analysis only made final sense in an evolutionary context.

Waddington's interests extended far beyond natural science and it was characteristic that he should not regard the cultural and political movements of his time as isolated from science or it from them. His public image was first created with the wartime Pelican *The Scientific Attitude* which, although necessarily burdened by contemporary problems, still makes interesting reading.

Conversation with Waddington was always stimulating. He lacked pomposity, always had some new preoccupation to expound, and treated the work of others with the same respect whether they were eminent or unknown, personal friend or personal foe. He will be missed.

D. R. Newth

THE recent death of my old friend and collaborator C. H. Waddington left me desolated by the break with the past that it entailed. But I must say that my primary feeling was one of great distress that he should have had to go just at this time when his wisdom is so much needed for the world. There are very few people of impeccable scientific standing and acknowledged genius who yet have a concern for ethics, for human society bettered and safeguarded, and for the forms of experience other than the natural sciences, together with such a calm, humane, generous and humorous spirit. I would have wanted at least a dozen more years of his judgment and leadership. His recent lectures on 'biological engineering', and the anti-science movement of the counter-culture, at the Royal Society and the Royal Society of Arts, made a great impression.

I knew him first in the twenties when I was starting research in Cambridge, and he was a member of the University Science Club, together with Gregory Bateson and other mutual friends of those generations. The period of our association covered just exactly the decade of the thirties, for the year in which my *Chemical Embryology* was published (1931) was the same year in which Otto Mangold and others, notably Wad himself, showed that the primary neural inductor in vertebrates

was stable to physicochemical denaturing agents. Out of this arose our collaboration in chemical embryology, studying what we used to like to call the 'morphogenetic hormones'—a conception still necessary today, although remarkably little progress has been made in the identification of these intermediate products of the genes.

Wad made a splendid collaborator. In intellectual grasp and analytical penetration I always felt he was gifted far beyond me. The great thing about him was that he was a man with whom it was impossible to quarrel. When one knew him only a little in those young days one might at first think him unduly reserved, even cold, with no room for the passionate at all; but this was quite a misconception, because throughout his life he enjoyed many deep friendships and attachments with people of very varying ages. At the same time he was singularly 'unflappable', a quality that stood him in good stead when in the field for geology or exploration. Always politically progressive but entirely without fanaticism, he would do everything he could to help younger people, and those who were refugees from their own lands.

Another aspect which I appreciated so much in him was his understanding of forms of experience other than that of the natural sciences, notwithstanding his fundamental and all-pervading deep rationality. In his case it was philosophy and the nature of aesthetic experience to which he was largely drawn, which might account in part for his marrying a brilliant architect; in my case it was always religion and theology, but we never had any disagree-

ments on such matters. Besides, we came together very closely on the ground of ethics, because we were both entirely convinced that the only ultimate salvation of humanity would lie in the control of the discoveries of the natural sciences by an ethic, a morality; the latter would have to be an entirely new one, congruent with the vast increases in human knowledge and the frightening powers over Nature which humanity was acquiring and would acquire. There has been no-one in my life whom I have liked and admired more, and I can only hope that his writings and his philosophical outlook will still influence younger people for many years to come.

Joseph Needham

John Wilfred Linnett, FRS, Master of Sidney Sussex College, Cambridge, and a noted theoretical chemist, died on November 7, at the age of 62.

JACK LINNETT was born on August 3, 1913, and educated at King Henry VIII School, Coventry, and St John's College, Oxford. After holding a Research Fellowship at Balliol he was made a Fellow of Queen's College, Oxford, in 1945. He was for twenty years first a Demonstrator then a Reader in the Inorganic Chemical Laboratory in Oxford, and was appointed Professor of Physical Chemistry at Cambridge in 1965. After a period as a Professorial Fellow at Emmanuel he was elected Master of Sidney Sussex College in 1970. He served as Vice-Chancellor at Cambridge from 1973–75, and had relinquished this arduous post only five

weeks before his death. He was president of the Faraday Division of the Chemical Society from 1971–73, and was to have become President of the Chemical Society in 1976. He had been a visiting professor at the Universities of Wisconsin, California, Cornell and Minnesota, and had strong ties with Malaysia and Singapore in his capacity as an External Examiner. His two books on valence theory are known to all undergraduates, and his 260 published papers covered such a variety of chemical topics that they stamp him as unique: infrared spectroscopy, combustion and flames, force constants, kinetics, catalysis, surfaces, wave mechanics. There are few aspects of physical or theoretical chemistry on which he has not left his mark. He was elected a Fellow of the Royal Society in 1955; had served the city of Oxford as a JP; and was an Honorary Fellow of Queen's and St John's Colleges, Oxford. In recent years he had become increasingly interested in the teaching of chemistry as an educational process in its own right, and devoted much time to communicating his thoughts on this topic to others. In spite of the enormous demands on his time, he was always accessible to undergraduates, research students, colleagues, staff and friends, and was willing to give advice and lend a hand whenever and wherever it was required. He died very abruptly on 7 November 1975, typically between giving a PhD oral and addressing the Royal Society, at the very height of a formidable career. It is difficult to assess how many people will be needed to fill the gap that he has left.

P. J. Wheatley

announcements

Awards

The French Academy of Science has awarded its main annual prize jointly to **S. Brenner** and **S. Benzer** for their achievements in the field of molecular genetics.

G. Barsky and **B. Ephrussi** have been awarded the **Paul Ehrlich Award** for their pioneering work on cell fusion.

The **1975 Albert Lasker Awards** are as follows: The **Clinical Medical Research Award** to **G. N. Hounsfield** and **W. Oldendorf**; A **Basic Medical Research Award** to **R. C. L. Guillemin** and **A. V. Schally**; Another **Basic Medical Research Award** to **F. Dixon** and **H. G. Kunkel**; The **Special Award** to **K. H. Beyer**, **J. M. Sprague**, **J. E. Baer** and

F. C. Novello; The **Public Service Award** goes to **J. Stein**.

Appointment

K. P. Duncan has been appointed a member of the **Medical Research Council**.

Reports and publications

Other countries

Publications du Centre National pour l'Exploitation des Océans (CNEO). Série: Rapports Scientifiques et Techniques, No. 21: *Isolément in situ d'Eau de Mer Naturelle dans des Encloses de Grand Volume: Application à l'Étude d'Une Eutrophisation Intéressante et Prospective*. Par Brigitte R. Berland, Daniel J. Bonin et Serge Y. Maestrini. Pp. 18. (Paris: CNEO, 1975.)

Smithsonian Contributions to Zoology, No. 192: *Review of Ochsenheimeriidae and the Introduction of the Cereal Stem Moth Ochsenheimeria vacuolella into the United States (Lepidoptera: Tineoidea)*. By Donald R. Davis. Pp. iii + 20. No. 193: *The Principal Larrea Bees of the Southwestern United States (Hymenoptera: Apoidea)*. By Paul D. Hurd, Jr., and E. Gordon Linsley. Pp. iii + 74. (Washington, DC:

Smithsonian Institution Press, 1975. For sale by US Government Printing Office.)

CERN — European Organization for Nuclear Research. CERN 75-11: *Polarized Particles for Accelerator Physicists*. By J. S. Bell. Pp. vii + 37. (Geneva: CERN, 1975.)

United States of the Interior: Geological Survey. Professional Paper 898: *Slump Blocks in the Atlantic Highlands of New Jersey*. By James P. Minard. Pp. iii + 24. (Washington, DC: Government Printing Office, 1974.) 95 cents.

Smithsonian Contributions to Zoology, No. 198: *Ctenuchid Moths of Ceramidia Butler, Ceramidiodes Hampson, and the Caca Species Group of Antichloris Hubner*. By William D. Field. Pp. iii + 45. (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.)

International Institute for Applied Systems Analysis. Annual Report—IIASA '74. Pp. xiv + 42. (Laxenburg, Austria: IIASA; London: The Royal Society, 1975.)

Coastal Water Research Project—Annual Report for the year ended 30th June 1975. Pp. v + 211. (El Segundo, California: Southern California Coastal Water Research Project, 1500 East Imperial Highway, 1975.)

United States Department of the Interior: Geological Survey. Professional Paper 837: *The Logic of Geological Maps, With Reference to Their Interpretation and Use for Engineering Purposes*. By David J. Varnes. Pp. iii + 48. \$3.50. Professional Paper 856: *Structure and Origin of the Koa Fault System, Kilauea Volcano, Hawaii*. By Wendell A. Duffield. Pp. v + 12 + plate 1. (Washington, DC: Government Printing Office, 1974 and 1975.)